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THE JAPANESE JOURNAL OF CANCER RESEARCH

Founded by K. YAMAGIWA and Continued by M. NAGAYO

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THE JAPANESE CANCER ASSOCIATION AND
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SEVENTH INTERNATIONAL CANCER CONGRESS

LONDON, ENGLAND July, 1958

Sponsored by **The International Union Against Cancer**

The Seventh International Cancer Congress will be held in London, England, July 6-12, 1958 under the Presidency of Sir Stanford Cade. Congress headquarters will be The Royal Festival Hall.

There will be two main sessions of the Congress: A. Experimental B. Clinical and Cancer Control. Special emphasis will be placed on Hormones and Cancer Chemotherapy, Carcinogenesis and Cancer of the Lung.

Proferred papers will only be considered if submitted with an accompanying abstract (not over 200 words) before October 1957 and if dealing with new and unpublished work.

The registration fee for the Congress will be £10 (ten pounds) or $30 (thirty dollars) and the latest date for registration without late fee will be January 1, 1958.

Registration forms and a preliminary program will be availabe early in 1957 on application to

The Secretary General
Seventh International Cancer Congress
45 Lincoln's Inn Fields
London, W. C. 2, England

[GANN, 48, 1—7 ; March, 1957]

EFFECTS OF X-IRRADIATION UPON THE NUCLEIC ACID CONTENT OF YOSHIDA SARCOMA*

TAKASHI MIURA, SHIGERU MORI, ITSUO KAWAMOTO
and SHUNSO NAGAI

Department of Radiology, Osaka University Medical School
(Director: Prof. T. Nishioka)

It is reported by many investigators[1]-[10] that desoxypentosenucleic acid (DNA) or nucleoprotein extracted from various animal tissues is depolymerized after exposure of a large dose of X-radiation.

Accordingly a fluctuation in nucleic acid (NA) content of various tissues or cells of living individuals may be expected when a therapeutic dose of X-radiation is applied upon them. Reports have recently been made on this subject by several investigators[11]-[17] not without some differences in their results.

By way of evaluating these results Nagai and his co-workers[18]-[20] have made a series of studies on this subject both quantitatively and histochemically, some of which have been already reported.

Among experimental tumors Yoshida sarcoma is so radiosensitive that remarkable morphological as well as cytochemical changes could be observed following X-irradiation of intermediate dose.[21]-[26] A quantitative measurement can be easily performed on NA and other substances in Yoshida sarcoma, because it is safely assumed that ascites tumor affords a homogeneous emulsion of tumor cells, as maintained by Klein et al.[27]-[29]

In the present paper, therefore, a chemical study has been made on NA content of the ascites of Yoshida sarcoma as a representative of malignant tumors, following whole-body X-irradiation.

MATERIALS and METHODS

As hosts of Yoshida sarcoma commercially available rats of both sexes weighing ca. 100 g. were fed under a certain conditions for at least 2 weeks before experiments. 5 to 8 days after intraperitoneal transplantation of the ascites of Yoshida sarcoma containing about $6\text{-}16\times10^6$ tumor cells to the animals, their ascites was employed for determining of NA before their death which was generally anticipated to occur on the 10-11 th day after transplantation.

* Presented at the 12 th Congress of Japan Radiological Society at Kumamoto on the 6 th of April, 1953.

Animals received a single whole-body irradiation of 500 r under 160 Kvp, 3 mA, filtration with 0.5 mm Cu and 0.5 mmAl, 23 cm target-animal-distance and 20r/min. The irradiation was done at the later stage of transplantation when relatively abundant ascites had accumulated, so that the ascites might be still significant in amount despite the influence of X-irradiation.

The peritoneum of hosts being opened under aether anesthesia, the ascites was aspirated with glass capillaries, with a minute care not to contaminate the ascites with bleeding.

After the NA fraction was extracted from the collected ascites by a modified Schneider's method[30], an extract was made out of the NA fraction by hot 5 per cent trichlor-acetic acid. Being reacted by Dishe's reagent, it was colorimetrically determined for DNA. Also it was processed by orcin-hydrochloric acid reaction to determine pentosenucleic acid (RNA) content. Both DNA and RNA values obtained were described by the microgram of their phosphorus.

On the other hand, a number of cells in the ascites was counted with Buerker's blood counting plate and a ratio of tumor cells to the total cells in the ascites was determined on stained smear specimens of the same ascites. A mean NA content per cell could be thus obtained from the NA content and the total number of cells in a certain quantity of the ascites.

Since the ratio of the tumor cells to the total cells was ranged 87-94 per cent a number of non-tumorous cells in the ascites seemed negligible.

A method was specifically contrived to make a homogeneous emulsion of the ascites cells of irradiated animals before mean NA content per cell was determined. Because, following irradiation the ascites specimen is subject to contamination with numerous cell debris giving much trouble in both determination and counting.

The method consists of repeated, low speed centrifugations (about 500 rpm) followed by washing of the diluted ascites with saline solution.

Results

A. The time course of ascitic NA content of Yoshida sarcoma following transplantation.

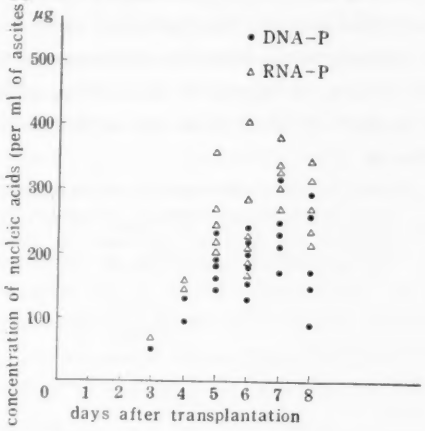
1) Ascitic NA concentration.

3, 4, 5, 6, 7, and 8 days following transplantation, the NA concentration of the ascites was investigated as illustrated in Figure 1. It was significant that the ascites NA level showed an increasing trend after transplantation, in the presence of considerable, individual variations.

2) Mean NA content per cell in the ascites.

Mean NA content per cell estimated from the data of 5, 6, 7, and 8 days after

Figure 1. The time course of ascitic nucleic acid concentration of Yoshida sarcoma after transplantation.



transplantation is summerized in Table 1. Both DNA-P and RNA-P values per cell remained almost constant, with a very slight decreasing trend following transplantation, ranging 1.16-0.94$\times 10^{-6}\mu$g and 1.44-1.32$\times 10^{-6}\mu$g respectively, in spite of a considerable, daily fluctuation of ascitic NA concentration.

The ratio of RNA to DNA remained almost constant centering around 1.4.

3) NA content in the liquid component of the ascites was determined after the cells were

Table 1. Mean nucleic acid content in the ascites after transplantation.

days after transplantation	No. of animals	DNA-P concentration (μg/ml)	RNA-P concentration (μg/ml)	DNA-P $\times 10^{-6}$ μg/cell	RNA-P $\times 10^{-6}$ μg/cell	$\frac{\text{RNA-P}}{\text{DNA-P}}$	$\frac{\text{Tumor cells}}{\text{total cells}}$ (%)
5	5	214	291	1.16	1.44	1.24	87
6	6	187	243	1.12	1.46	1.42	91
7	5	218	331	0.91	1.31	1.44	94
8	5	176	242	0.94	1.32	1.40	92

eliminated by centrifugation (Table 2). The elapse of time following irradiation or cell concentration of the original ascites gave little difference in the determination. The determined value was so low about 2 per cent of the total NA content in the ascites, that any possible influence on estimating mean DNA content per cell was negligible.

Table 2. Mean DNA-P concentration in the liquid component of the ascites after transplantation.

days after transplantation	cell-concentration $\times 10^6$ cells per ml	DNA-P μg/ml
5	151	2.23
6	230	2.89
7	322	2.64
8	252	2.59

B. Ascitic NA content of Yoshida sarcoma following X-irradiation.

1) Ascitic NA concentration.

Because of considerable individual variation in ascitic NA concentration which

were obtained at a corresponding period after transplantation, it seemed more reasonable to observe the same animal before and after irradiation. But it was impossible to take a ascites specimen from a single animal sufficient enough to cover two experiments before and after irradiation. The authors' next best method was to conjecture ascitic NA concentration before irradiation, on the basis of count of a small quantity of the ascites, in regard to mean value of NA content per cell. In order to count a number of the cells in the ascites only a small quantity of the specimen was sufficient.

The data summarized were illustrated in Figure 2, in which the control ascitic NA concentration estimated from a number of cells in the ascites was standardized as 100 per cent and ascitic NA concentrations actually investigated following irradiation were represented against the percentage.

A definite and parallel fluctuation of both DNA and RNA concentrations was observed following irradiation, indicating a slight depression after 1 hour, a slight elevation after 3 hours and another marked depression ranging over 30 per cent of standard value after 24 as well as 48 hours.

Figure 2. The time course of ascitic nucleic acid concentration following X-irradiation of 500r. The data are expressed as percentages of the control values.

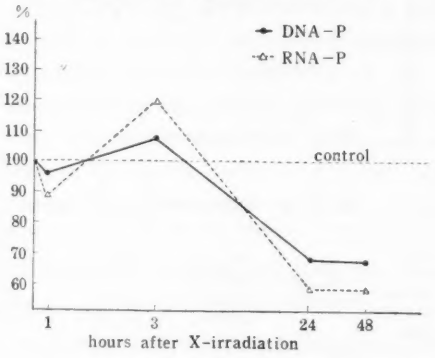


Table 3. Mean nucleic acid content per cell in the ascites following X-irradiation of 500r.

hours after irradiation	No. of animals	DNA-P $\times 10^{-6}$ μg/cell	RNA-P $\times 10^{-6}$ μg/cell	$\frac{\text{RNA-P}}{\text{DNA-P}}$
1	5	0.967	1.32	1.4
3	5	0.966	1.55	1.6
24	5	0.958	1.53	1.6

2) Mean NA content per cell in the ascites.

Mean NA content per cell in the ascites was investigated at 1, 3, and 24 hours after irradiation as summerized in Table 3. The level of DNA per cell was found to be very stable, while that of RNA unstable.

Discussion

Mean NA content per cell of Yoshida sarcoma was reported by G. Klein who has studied on various animal tumors of the ascites type. The results of the present study are in general accord with those of Klein in spite of considerable differences in cell concentration, percentage of tumor cells, and of polymorph as well as mitotic cells in the ascites.

The minimal declination of NA content per cell of the ascites occurring when plotted against the time after transplantation as presented in Table 2 agreed with the results obtained by Tobioka and Ueoka[31] in their study on the metabolism of NA of Yoshida sarcoma. This occurred despite the fact that a striking difference was found between values of NA content obtained by those and the present authors.

Observations in the present study on alterations of NA content of Yoshida sarcoma due to X-irradiation were performed by measuring NA concentration in the ascites and mean NA content per cell. However, as control an estimated value was employed because investigation on the ascites before irradiation was impossible to perform. Though estimates may be inadequate as exact controls a definite degree of their alterations following irradiation can be observed.

From the results obtained in the experiments it was found that DNA content per cell remained unchanged after irradiation unless the cells were destroyed as a result of irradiation. On the contrary RNA content per cell showed some increase.

Many microspectrophotometric researches[12][16][32][33][34] in this field were reported with contradictory results. Quantitative investigation of the total thymus DNA content following β-ray irradiation was reported to decrease by Harrington et al,[35] but the change in the DNA content per cell is in accord with the present authors' results on the ascites of Yoshida sarcoma. The total NA content of various organs and tissues was reported by most authors to decrease following irradiation. This reduction in the total NA content is assumed to be caused not by a uniform decrease of NA content per cell, but rather by destruction of a large number of tissue cells with the NA content per surviving cell remaining unchanged.

Recently studies making use of NA content as an index of cell proliferation have become popular. If it may be sure at all that destroyed cells play a main role only in a reduction in quantity of NA content of a certain organ or tissue following irradiation, quantitative determination of the rate of tissue destruction following irradiation may be possible by means of quantitative determination of NA content. This idea may be useful with reference not only to the radiation effect but also to the effects of chemical and antibiotic substances against cancer.

Summary

1) Ascitic NA content of Yoshida sarcoma was measured by means of quantitative investigation of ascitic NA concentration and NA content per cell. From the result obtained in the experiments it was found that both DNA and RNA contents per cell remained within a definite range during the usual proliferation

of Yoshida sarcoma, but with aging of the sarcoma there was a diminution in these contents, as opposed to ascitic NA concentration which was found to increase as time wore on, though varying greatly according to individuals.

2) At twenty-four hours following single whole-body exposure to 500r of X-radiation ascitic DNA and RNA concentrations of Yoshida sarcoma were found to be decreased considerably, but the DNA content per cell was found to remain unchanged and RNA content per cell to be increased slightly so far as the surviving cells in the ascites were concerned.

Acknowledgments

The authors are indebted to A. Shibatani for his advice in the determination of the nucleic acid.

References

1) Sparrow, A. H., and F. M. Rosenfeld: Science, 104: 245-246, 1946.
2) Errera, M.: Cold Spring Harbor Symposia Quant. Biol., 12 ; 60-63, 1947.
3) Taylor, B., J. P. Greenstein and Hollaender : ibid., 237-246, 1947.
4) Scholes, G., G. Stein and J. Weiss: Nature, 164 : 709-710, 1949.
5) Limpelos, G., and W. A. Mosher: Am. J. Roentg., 63 : 681-690 ; 691-700, 1950.
6) Butler, J. A. V. and K. A. Smith: J. Chem. Soc., 1950, 3411-3418.
7) Butler, J. A. V., and B. E. Conway: ibid., 1950, 3418-3421.
8) Conway, B. E., L. Gilbert and J. A. V. Butler: ibid., 1950, 3421-3425.
9) Press, E. H. and, J. A. V. Butler: ibid., 1952, 626-631.
10) Conway, B. E. and J. A. V. Butler: ibid., 1952, 834-838.
11) Gregoire, P. E., and Ch. Gregore: Arch. Int. Med. Exptl., 9 : 283-316, 1934.
12) Mitchell, J. S.: Brit. J. Expt. Path. 23 : 285-295; 296-308; 309-313, 1942.
13) Koller, P. C.: Nature, 151 : 244-246, 1943.
14) Euler, H. V., and Rönnestann-Säberg: Arkiv. Kemi. Mineral Geologi, 23 A, No. 11, 1946.
15) Stowell, R. E.: Symposia Soc. Exptl. Biol., 1 : 190-206, 1947.
16) Ely, J. O., and M. H. Ross: Cancer Res. 8 : 285-294, 1948.
17) Hamazaki, S.: Physiology and Pathology of Cell Nuclei. Nagai Co. Osaka, 1954.
18) Nagai, S.: Jap. Med. J., 1537 : 27-30, 1953.
19) Nagai, S., H. Matsuda, K. Akita and T. Kasue: Med. J. Osaka Univ., 5 : 749-762, 1954.
20) Akita, K.: Osaka Daigaku Igaku Zassi. 8 : 199-204, 1956.
21) Nakatsuka, H., and Y. Kishi: Nippon acta radiol., 8 (1) : 30, 1948.
22) Muta, S.: ibid., 10 (1) : 30-35, 1950; 11 (3/4) : 35, 1951.
23) Shibata, M.: ibid., 11 (8) : 1-9, 1951.
24) Note, R.: ibid., 12 (2) : 65-77, 1952.
25) Ono, T.: ibid. 12 (6) : 8-9; 10-15; 12 (7) : 7-9, 1952.
26) Kike, T.: ibid., 12 (7) : 3-6, 1952.
27) Klein, G.: Cancer, 3 : 1052-1061, 1950.
28) Goldberg, L., E. Klein and G. Klein: Explt. Cell Res. 1 (4) : 543-570, 1950.

29) Klein George: Explt. Cell Res. 2 (3) : 518-573, 1951.
30) Egami, F.: Nucleic Acids and Nucleoproteins. Kyoritsu Press. Tokyo, 1951.
31) Tobioka, M., and K. Ueoka: Gann, 41 : 107, 1950; 42 : 113, 1951; 43 : 227, 1952.
32) Stowell, R.E.: Cancer Res. 5 : 169-178, 1945.
33) Harrington, N.J., and R.W. Koza: Biol. Bull. 101 : 138-150, 1951.
34) Moses, M.J., R. Dubow and A.H. Sparrow: J. Natl. Cancer Inst. 12 : 232-235, 1951.
35) Harrington, H., and P. Lavick: Fed. Proc. 10 : 194-195, 1951.

要　旨

吉田肉腫の核酸含量に及ぼすX-線照射の影響

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大阪大学放射線医学教室（主任：西岡時雄教授）

1) 吉田肉腫の腹水の核酸濃度と細胞1個あたりの平均核酸含量を化学的定量法によって測定した。吉田肉腫の正常増殖過程においては核酸濃度は個体によって著しい差があるがほぼ経過とともに増加し末期では増加しない。細胞1個あたり平均核酸含量は DNA-P, RNA-P ともに軽度の減少を示す傾向はあるがほとんど差がないとみなし得る。

2) X-線 500 r 全身照射後の値を測定するに核酸濃度は DNA-P, RNA-P ともに照射後24 時間で著しい減少を示した。細胞 1 個あたり平均核酸含量は細胞が破壊せられていない限りにおいては DNA-P 量には変動はみられず RNA-P 量は軽度に増加しているようである。

[GANN, 48, 9~14; March, 1957]

RELATIONSHIP BETWEEN THE HEREDITARY TUMORS AND TRANSITION METALS IN DROSOPHILA MELANOGASTER

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(Department of Genetics, Faculty of Medicine, Osaka University)

INTRODUCTION

The majority of the hereditary tumors in *Drosophila melanogaster* appears as the malanotic mass floating in the body fluid, or the melanotic tissue inserted in tissues of the fat body, the hypoderm, the trachea, and the intestine, and it appears at the outset of the third instar larva and shows pronounced growth up to the late pupal period.

Kikkawa *et al*,[1] and Abe[2,3] reported that in the black pigment in various organs of animals Fe and Cu were contained more abundantly than in the yellow and the white pigments, and they suggested that these transition metals might play some important parts in the process of the melanin formation. Kuroda and Tamura[4,5] found that the melanotic growth of tumors in *D. melanogaster* was markedly accelerated by the addition of Cu, when these tumors were cultured in the synthetic medium composed of amino acids, vitamins, glucose, inorganic salts, and ribonucleic acid. They[6] also showed that the melanotic growth of these tumors was inhibited by phenyl-thio-carbamide (P.T.C.) which had an ability to combine with various metals, especially with Cu. These results seem to suggest differences in the metal contents between the melanotic tumorous tissues and the normal ones. In the present study, the metal contents of the tumorous strains were compared with those of the non-tumorous strains.

MATERIALS AND METHODS

Two melanotic tumorous strains, *v tu* and *st tu*, and a non-melanotic tumorous strain, *tu-h*, were used as materials having hereditary tumors. *v*, *st*, and *Oregon* (Wild) strains were used as controls of the above tumorous strains respectively.

The first day's pupae after the pupation, which had been aseptically cultured in the food mixture composed of malted rice, sugar, peptone, yeast powder, and agar, were collected and washed repeatedly in double-distilled water. The pupae dried for 24 hours at 70°C. in an electric oven and were weighed. 200 mg of the dried samples were completely ashed in a crucible below 300°C by the dry oxidation method. After dissolving the ash in a small quantity of 6 N hydrochloric acid, the solution was condensed and the quantities of iron and copper in this

solution were measured quantitatively by Sandell's method.[7] Hydroquinone and *o*-phenanthroline were used for the measurement of iron, and diethyl-dithio-carbamate was used for the estimation of copper.

Experimental Results

1) Comparison of amounts of metals between the *v tu* and the *v* strains.

The amounts of iron and copper contained in the first day's pupae after pupation in the *v tu* and the *v* strains were shown in Table 1.

Table 1. Amounts of Fe and Cu (p.p.m.) contained in the first day's pupae after pupation in the *v tu* and the *v* strains.

Exp. No.	*v tu*		*v*	
	Fe	Cu	Fe	Cu
1	294	211	270	199
2	369	225	273	154
3	320	198	308	129
Mean	328	211	284	161

The average amount of iron contained in *v tu* pupae was 328 p.p.m., greater than that contained in *v* pupae, 284 p.p.m. The amount of copper contained in *v tu* pupae was 211 p.p.m., greater than that contained in *v* pupae, 161 p.p.m. It is assumed that the melanotic tumorous pupae contained more amounts of iron and copper as compared with the non-tumorous pupae.

2) Comparison of amounts of metals between the *st tu* and the *st* strains.

The quantities of iron and copper contained in the first day's pupae after pupation in the *st tu* and the *st* strains were shown in Table 2.

Table 2. Amounts of Fe and Cu (p.p.m.) contained in the first day's pupae after pupation in the *st tu* and the *st* strains.

Exp. No.	*st tu*		*st*	
	Fe	Cu	Fe	Cu
1	282	346	140	356
2	209	386	131	340
3	201	340	163	342
Mean	231	357	145	346

As shown in the above table, the average amount of iron contained in *st tu* pupae was 231 p.p.m., showing a higher level than that contained in *st* pupae, 145 p.p.m. A greater amount of copper was also detected in *st tu* pupae than in *st* pupae. The greater quantity of copper contained in the *st tu* strain seems to

be due to having *st* gene which corresponds to the greater amount of copper than that contained in *v* pupae, as shown in Tables 1 and 2. It is clear that the melanotic tumorous strains had greater amounts of iron and copper than that in the non-tumorous strains.

3) Comparison of amounts of metals between the *tu-h* and the *Oregon* strains.

The amounts of iron and copper contained in the first day's pupae after pupation in the *tu-h* and the *Oregon* strains were shown in Table 3.

Table 3. Amounts of Fe and Cu (p.p.m.) contained in the first day's pupae after pupation in the *tu-h* and the *Oregon* strains.

Exp. No.	*tu-h*		*Oregon*	
	Fe	Cu	Fe	Cu
1	381	242	167	159
2	391	265	152	154
3	404	301	140	185
Mean	392	269	153	166

As shown in Table 3, the average amount of iron contained in *tu-h* pupae was 392 p.p.m., representing a greater quantity as compared with that in *Oregon* pupre, 153 p.p.m. The average amount of copper contained in *tu-h* pupae was 269 p.p.m., showing a greater amount than that in *Oregon* pupae. It is assumed that the non-melanotic tumorous strain had also greater amounts of iron and copper than that in the non-tumorous strain.

Discussion

The amounts of iron and copper contained in the melanotic tumorous strains, *v tu* and *st tu*, were shown to be greater than those in the non-tumorous strains, *v* and *st*. A greater amount of iron contained in the *v tu* strain than that in the *st tu* strain seems to correspond to a greater amount of iron contained in the *v* strain than that in the *st* strain. A greater amount of copper contained in the *st tu* strain than that in the *v tu* strain also seems to correspond to a greater amount of copper contained in the *st* strain than that in the *v* strain. The relation of the amounts of copper in both *st* and *v* strains coincides with results reported by Kikkawa *et al.*,[1,8] who detected greater amounts of copper in the *st* strain. Also in the non-melanotic tumorous strain, *tu-h*, greater amounts of iron and copper was detected as compared with that in the non-tumorous strain, *Oregon*. Therefore, three hereditary tumorous strains had greater amounts of iron and copper than those in the non-tumorous strains.

Heath and Liquier-Milward[9] reported that in the sarcoma and carcinoma in mice, the malignant tissue took up more of ^{65}Z/unit weight of tissue than the

normal tissue supporting tumor growth. It is interesting that this result may represent the importance of the transition metals contained in the tumorous tissues.

Mittler,[10] Glass and Plaine,[11] Plaine and Glass,[12] and Kanehisa[13] reported that the incidence of the melanotic tumors in *D. melanogaster* was increased by addition of tryptophan to the culture media. This fact seems to show that tryptophan metabolism may be related to the incidence of the melanotic tumor. Kanehisa[14] also found that the incidence of the melanotic tumor in *D. melanogaster* was increased by combining the tumorous gene with *v*, *cn*, or *st* genes which are known to block the tryptophan metabolism. This result seems to indicate that tryptophan or its derivatives accumulated in these mutants may have the effect of increasing the incidence of the melanotic tumors.

Doi and Abe[15] showed that the amounts of iron and copper increased and that of nickel decreased in the *white-1* mutant eggs laid by moths of the silkworm, *Bombyx mori*, which received an injection of 3-hydroxykynurenine in their early pupal stage. It is also assumed that in the hereditary tumors, the amount of iron accumulated in the body was increased correspondingly to the increase of tryptophan or its derivatives.

Kuroda and Tamura[4,6] found by means of the tissue culture that the melanotic growth of tumors in *D. melanogaster* was accelerated by addition of copper in the synthetic culture media. In anticipation of future work, we want to know how these transition metals are related to the formation of the hereditary tumors.

Summary

1) The amounts of metals contained in the first day's pupae after pupation in two melanotic tumorous strains, *v tu* and *st tu*, and a non-melanotic tumorous strain, *tu-h*, in *Drosophila melanogaster* were determined to compare with those contained in non-tumorous strains, *v*, *st*, and *Oregon*, respectively.

2) The greater amounts of iron and copper were detected in the melanotic tumorous strains, *v tu* and *st tu*, as compared with the non-tumorous strains, *v*, and *st*, respectively.

3) The amounts of iron and copper contained in the non-melanotic tumorous strain, *tu-h* was also shown to be greater than those in the *Oregon* strain.

4) The relation of the formation of the hereditary tumors in *D. melanogaster* to the transition metals and tryptophan metabolism was discussed.

Acknowledgements

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work.

References

1) Kikkawa, H., Ogita, Z., and Fujito, S.: Nature of pigments derived from tyrosine and tryptophan in animals. Science, Vol. 121, 43-47, 1955.

2) Abe, K.: The relation of pigment to transition metals in mice. Med. J. Osaka Univ., Vol. 6, 343-345, 1955.

3) Abe, K.: Relationship between hair-colors and absorbability of transition metals in various organs of mice. Med. J. Osaka Univ., Vol. 6, 605-617, 1955.

4) Kuroda, Y. and Tamura, S.: The tissue culture of tumors in *D. melanogaster*. I. The nature of the melanotic tumors and the effect of the metamorphic hormone upon the melanotic growth of tumors. (In Japanese). Zool. Mag., Vol. 64, 380-384, 1955.

5) Kuroda, Y. and Tamura, S.: The tissue culture of tumors in *D. melanogaster*. III. The effects of Cu-ion upon the melanotic growth of tumors. (In Japanese). Zool. Mag., Vol. 65, 11-15, 1956.

6) Kuroda, Y. and Tamura, S.: The tissue culture of tumors in *D. melanogaster*. II. The effects of phenylthiocarbamide upon the melanotic growth of tumors. (In Japanese). Zool. Mag., Vol. 65, 219-222, 1956.

7) Sandell, E. B.: Colorimetric determination of trace of metals. Publishing Incorporation N. Y. New York, 1950.

8) Kikkawa, H., Ogita, Z., and Fujito, S.: Studies on the pigments derived from tryptophan in insects. Proc. Japan Acad., Vol. 30, 30-35, 1954.

9) Heath, J. C., and Liquier-Milward, J.: The distribution and function of zinc in normal and malignant tissues. I. Uptake and distribution of radioactive zinc, ^{65}Z. Biochem. et Biophy. Acta, Vol. 5, 404-415, 1950.

10) Mittler, S.: Influence of amino acids upon incidence of tumors in tu^{50j} stock of *D. melanogaster*. Science, Vol. 116, 657-659, 1952.

11) Glass, B., and Plaine, H. L.: Genetic control of tryptophan metabolism in *Drosophila*. Amino Acid Metabolism, (Johns Hopkins Press), 940-944, 1955.

12) Plaine, H. L., and Glass, B.: Influence of tryptophan and related compounds upon the action of a specific gene and the induction of melanotic tumors in *Drosophila melanogaster*. J. Genet., Vol. 53, 244-261, 1955.

13) Kanehisa, T.: A relation between the incidence of the melanotic tumor and the tryptophane metabolism in *Drosophila melanogaster*. (In Japanese). Japan J. Genet., Vol. 30, 170-171, 1955.

14) Kanehisa, T.: Relations of some eye-color genes to a melanotic tumor in *Drosophila melanogaster*. (In Japanese). Japan J. Genet., Vol. 29, 158, 1955.

15) Doi, K., and Abe, K.: Relation between egg-colors and amounts of transition metals in silk worm. I. Amounts of transition metals contained in *w-1* eggs colored by injection of 3-hydroxykynurenine. (In Japanese). Med. Biol., Vol. 37, 197-199, 1955.

要　旨

猩々蠅の遺伝性腫瘍と重金属との関係

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(大阪大学医学部遺伝学教室)

1. キイロショウジョウバエ (*D. melanogaster*) の2つのメラニン性腫瘍系統 ***v tu*** 及び ***st tu*** と非メラニン性腫瘍系統 ***tu-h*** の化蛹第1日目の蛹の金属含有量の定量を行い，それぞれの対象系統 ***v***, ***st***, 及び ***Oregon*** と比較した。

2. メラニン性腫瘍系統 ***v tu*** 及び ***st tu*** ではそれぞれ ***v*** 及び ***st*** に比較して著しく多量の鉄及び銅が検出された。

3. 非メラニン性腫瘍系統 ***tu-h*** においても ***Oregon*** に比較して多量の鉄及び銅が検出された。

4. ショウジョウバエにおける遺伝的腫瘍の形成と重金属及びトリプトファン代謝との関連が論議された。

[GANN, 48, 15~38; March, 1957]

STUDY ON THE ANTICANCEROUS ACTION OF UNSATURATED SEVEN MEMBERED RING STRUCTURE, ESPECIALLY COLCHICINE DERIVATIVES

(With Plates I and II)

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INTRODUCTION

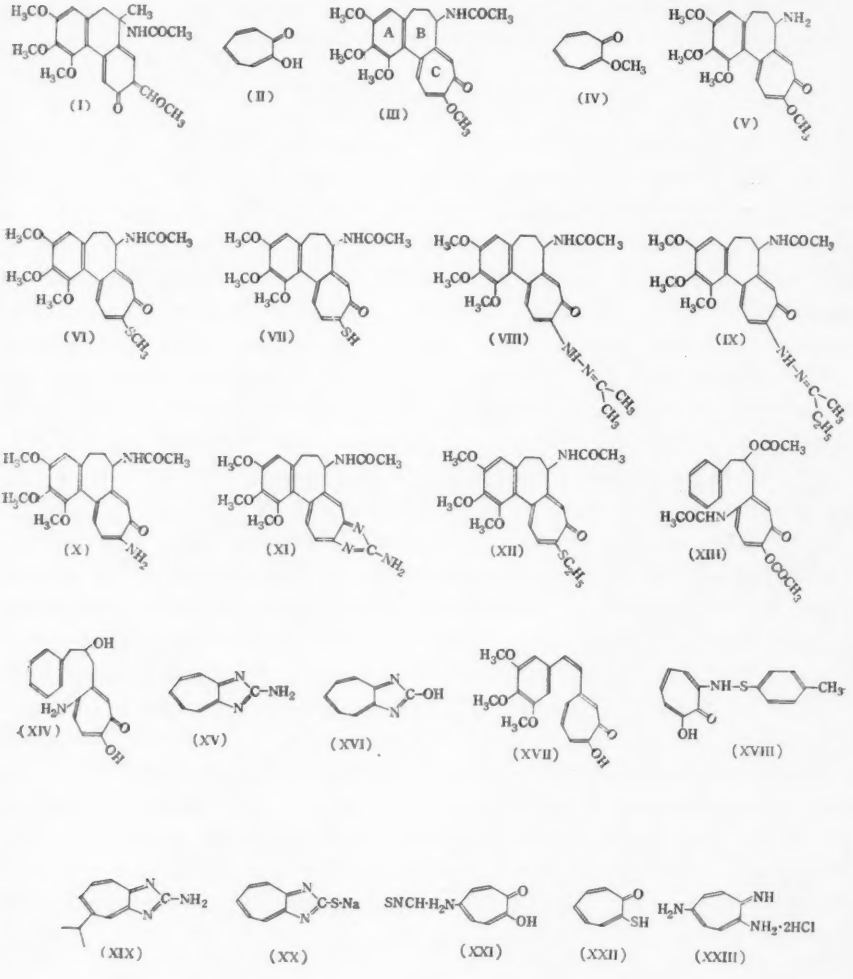
Colchicine has been used since an early period of medical history. Knowledge of its usage and nature may be divided into three stages. In the first stage, which may be called the classical stage, it was thought to be a placebo for gout and its alkaloid present in the corm, seeds and flowers of Colchicum autumnale was used. The second stage began with the reports that it was poisonous agent against cell division by Pernice[1], Amoroso[2] and Lits[3]. Thereafter, many papers on experimental and clinical subjects about colchicine were published. However, since colchicine was very poisonous, it was not a successful agent for the purpose of chemotherapy for human cancer. The third stage began with the study of the effect of colchicine-like substances. Brues and Cohen[4] reported the effect of 10 colchicine-like substances on regenerating liver in experimental animals. In 1945, Dewar[5] rewrote the formula of colchicine (I) which was proposed by Windaus[6], claiming that colchicine possessed unsaturated seven membered ring structure. In 1950, Nozoe[7], Doering[8], Cook[9] and Haworth[10] were successful in synthesizing tropolone (II), independently. Subsequently it was comfirmed that colchicine has a chemical formula shown in (III). The effect of many colchicine derivatives were reported by Santavy[11], Ullyot[12] and Leiter et al.[13][14]. On the other hand, Katsura and his associates[15][16][17] carried out experiments on many tropolone derivatives, especially studies on C ring structure were performed. According to these studies, tropolone was found to have no effect by itself but tropolone methyl ether (IV) had slight colchicine-like effect in the lethal doses, while its action was much inferior to colchicine. Sato[18], disclosed that it was hard to obtain the so-called colchicine-like effect with a single ring compound of tropolone in his experiment. Therefore it may be significant to test the effect of poly-membered ring structure including tropolone and new colchicine derivatives on tumor cells.

EXPERIMENT (1)

Screening Test on Yoshida Sarcoma

The effect of 20 compounds (Fig. 1. and Table 1. III, V-XXIII) with unsaturated seven membered ring structure, which are chiefly colchicine derivatives, on Yoshida sarcoma was studied. The technique and assay of the effect on sarcoma cells were those of Yoshida's screening test method, and the aceto-gentian violet was used for the stain. The administered concentration was studied in many cases

Fig. 1.



in dosage levels of 5 mg, 2 mg, 1 mg, 0.5 mg, 0.2 mg, 0.1 mg, and so on per 100 g of body weight.

Table 1.

	Vehicle
(I) Colchicine (Windaus)	water
(II) Tropolone	
(III) Colchicine	
(IV) Tropolone methyl ether	
(V) Trimethylcolchicinic acid methyl ether (*)	water
(VI) Thiocolchicine	p. g.
(VII) Thiocolchiceine	p. g.
(VIII) Condensation product of colchiceine-hydrazid with acetone	p. g.
(IX) Cndensation product of colchiceine-hydrazid with methyl ethyl ketone	p. g.
(X) Colchiceinamide	p. g.
(XI) Condensation product of colchicine with guanidine	p. g.
(XII) S-ethyl thiocolchiceine	p. g.
(XIII) 5-Amino-4-(β-hydroxy-γ-phenyl propyl) tropolone triacetate	p. g.
(XIV) 5-Amino-4-(β-hydroxy-γ-phenyl propyl) tropolone	p. g.
(XV) 2-Amino-1,3-diazazulene	p. g.
(XVI) 2-Hydroxy-1, 3-diazazulene	water
(XVII) β-Trimethoxy-styryl-tropolone	suspension
(XVIII) 3-(p-tolyl-sulfonyl) aminotropolone	suspension
(XIX) 2-Amino-5-isopropyl-1, 3-diazazulene	p. g.
(XX) 2-Mercapto-1, 3-diazazulene Na salt	p. g.
(XXI) 5-Amonotropolone HCNS salt	p. g.
(XXII) Mercaptotropone	p. g.
(XXIII) Diaminotroponimine HCl salt	water

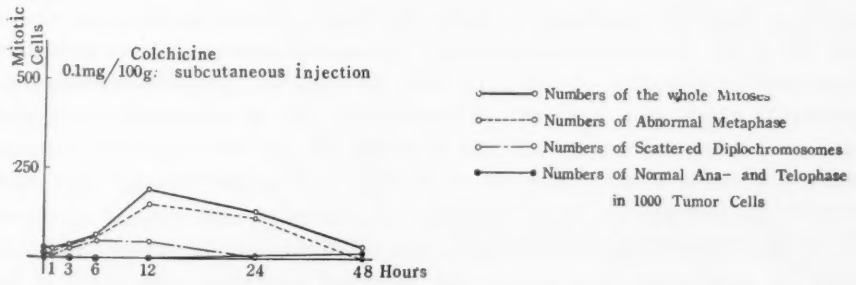
Indication: (*) ..... d-tartrate derivative used.
p. g. ..... propylene glycol

Those which showed influence on the tumor cells were as follows.

1) Colchicine (III).[19]

Scattered diplochromosomes which were observed one hour after subcutaneous injection of 0.1 mg (approximately 1/2 M.L.D.) (Fig. 2.) of this substance reached

Fig. 2. III, a.



the maximum at the 6th hour, and occupied most of the accumulated metaphase. Though the accumulation of metaphase reached the maximum at the 12th hour, scattered diplochromosomes did not increase after the 6th hour, and abnormal

metaphase such as the so-called star metaphase or distorted star metaphase, ball metaphase, etc., increased. Though these changes could be observed at the 24th hour, scattered diplochromosomes could hardly be seen. Ana- and telophase which did not appear up to 12 hours after injection started to appear at the 24th hour. In the resting tumor cells, the extracellular outflow of cytoplasma was observed 1-3 hours after injection, which decreased with lapse of time and was replaced by remarkable increase of caryolysis, caryorexis, etc. In cases of subcutaneous injection of 0.05 mg (approximately 1/4 M.L.D.), colchicine-mitose were slightly observed at the 3rd and 6th hour, while ana- and telophase did not disappear.

2) Trimethylcolchicinic acid methyl ether d-tartrate (T.M.C.A. methyl ether d-tartrate) (V).[12)13)]

With subcutaneous injection of 2 mg (approximately 1/2.5 M.L.D.) (Fig. 3.) of

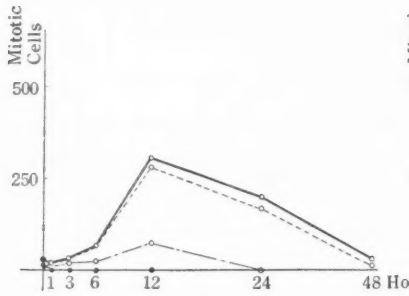


Fig. 3. V, a.
Trimethylcolchicinic acid methyl ether d-tartrate 2 mg/100 g subcutaneous injection.

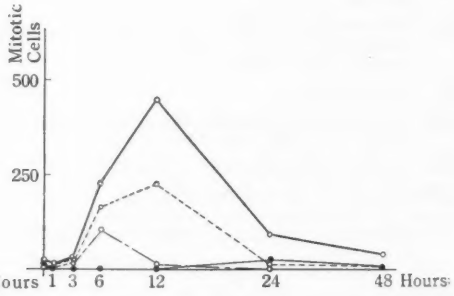


Fig. 4. V, b.
Trimethylcolchicinic acid methyl ether d-tartrate 1 mg/100 g subcutaneous injection.

this substance, the destruction of metaphase which appeared from one hour after injection started to accumulate from the 6th hour, and reached the maximum at the 12th hour. At this time scattered diplochromosomes and necrobiotic metaphase were both conspicuous. At the 24th hour no scattered diplochromosomes were observed, which were replaced with increasing rate of necrobiotic metaphase. Ana- and telophase which were not observed till the 12th hour after injection started to appear slightly after the 48th hour, and normal mitoses proceeded. Only slight prolongation of life was observed against controls without injections. With subcutaneous injection of 1 mg of this substance (approximately 1/5 M.L.D.) (Fig. 4.), accumulations of normal and abnormal metaphase were both at the maximum at the 12th hour, but scattered diplochromosomes were seen at most at the 6th hour. The findings of destruction became little at the 24th hour. Ana- and telophase both started to disappear one hour after injection, and at the

12 th hour abnormal ana- and telophase which showed marked difference of number of chromosomes despite division into dipolar, or picture with polypolar appeared.

With subcutaneous injection of 0.5 mg (approximately 1/10 M.L.D.) (Fig. 5.), the

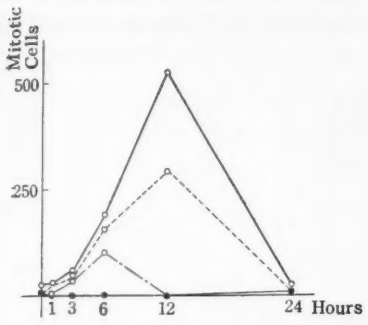


Fig. 5. V, c.
Trimethylcolchicinic acid methyl ether d-tartrate 0.5 mg / 100 g subcutaneous injcetion.

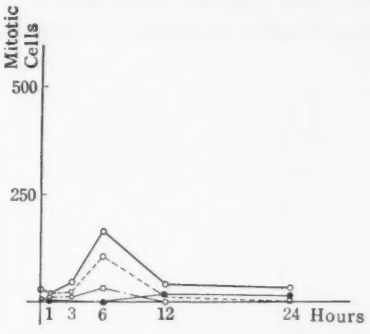


Fig. 6. V, d.
Trimethycolchicinic acid methyl ether d-tartrate 0.2 mg/100g subcutaneous injection.

whole picture followed that of 1 mg in general, but the degree of destruction was slight. Abnormal metaphase which was seen up to 12 hours disappeared at the 24 th hour. In case of subcutaneous injection of 0.2 mg of this substance (approximately 1/25 M.L.D.) (Fig. 6.), the effect markedly decreased, and although scattered diplochromosomes were observed up to the 6 th hour, it was 1 %–2 % of the tumor cells. Ana- and telophase began to recover at the 6 th hour, and

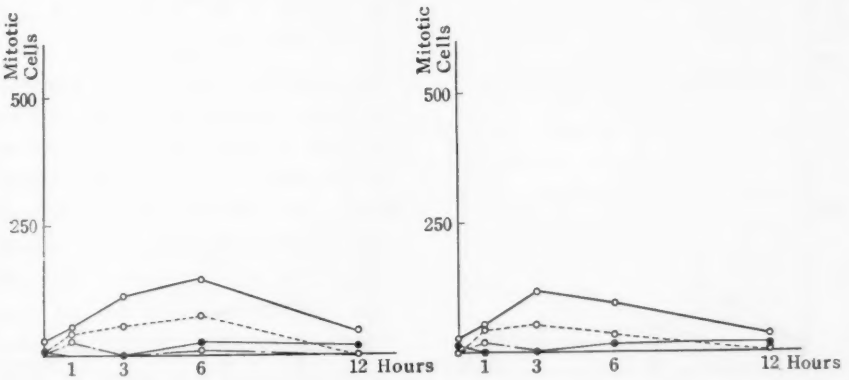
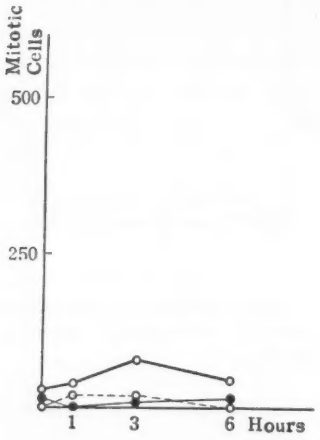


Fig. 7. V, e.

Trimethylcolchicinic acid methyl ether d-tartrate 0.1 mg/100g subcutaneous injection.

Trimethylcolchicinic acid methyl ether d-tartrate 0.1 mg / 100 g subcutaneous injection.

returned to normal by the 12th hour. With subcutaneous injection of 0.1 mg (approximately 1/50 M.L.D.) (Fig. 7.), abnormal metaphase was observed up to the 6th hour. Scattered diplochromosomes were, however, seen at most at the first hour and only few were seen 3 hours later. Ana- and telophase both recovered completely to normal at the 6th hour. With subcutaneous injection of 0.05 mg



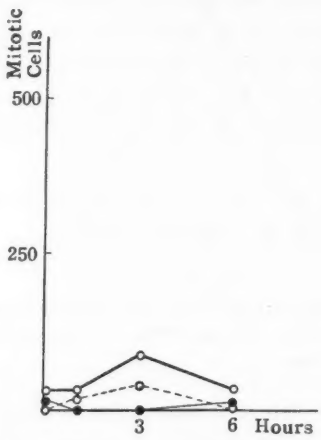
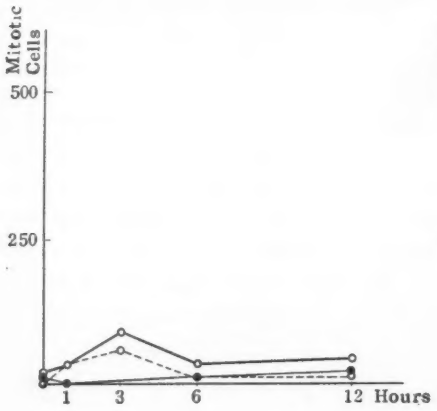


Fig. 8. V, f.

Trimethylcolchicinic acid methyl ether d-tartrate 0.05 mg / 100 g subcutaneous injection.

Trimethycolchicinic acid methyl ether d-tarate 0.05 mg / 100 g subcutaneous injection.



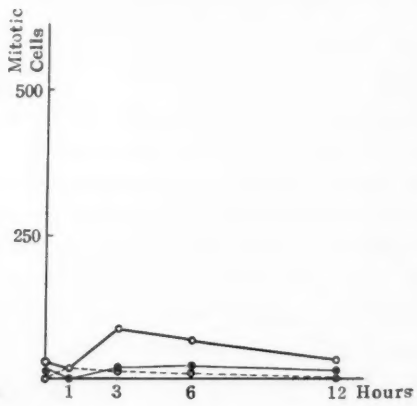


Fig. 9. V, g.

Trimethylcolchicinic acid methyl ether d-tartrate 0.05 mg/100 g intraperitoneal injection.

Trimethylcolchicinic acid methyl ether d-tarate 0.02 mg / 100 g intraperitoneal injection.

(approximately 1/100 M.L.D.) (Fig. 8.), accumulation of abnormal metaphase was slight. Most of them were star metaphase. Ana- and telophase disappeared within 1–3 hours which was followed with recovery to normal. No influence was observed with subcutaneous injection of 0.02 mg (approximately 1/250 M.L.D.). Destruction of resting cells was observed remarkably with dosage of 2 mg, followed with slighter effect with decrease of quantity.

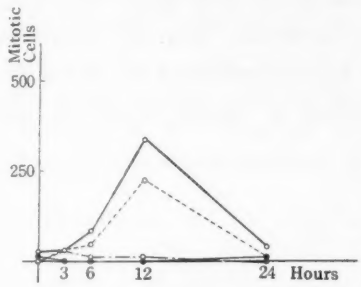


Fig. 10. V, h.
Trimethylcolchicinic acid methyl ether d-tartrate 0.2 mg/100 g intraperitoneal injection.

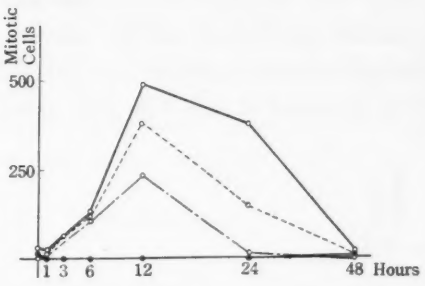


Fig. 11. VI, a.
Thiocolchicine 0.2 mg/100 g subcutaneous injection.

In case of intraperitoneal injection, some influence was seen with 0.02 mg (approximately 1/250 M.L.D.) (Fig. 9.), while the effect continued up to 12 hours with dosage of 0.2 mg (Fig. 10.). Scattered diplochromosomes were very slightly seen one hour after the injection with 0.05 mg. Ana- and telophase began to appear by the 12th hour with 0.1 mg, by the 6th hour with 0.05 mg, and by the 3rd hour with 0.02 mg.

3) Thiocolchicine (VI).[20)21)]

Colchicine-like effect which appeared an hour after subcutaneous injection of 0.2 mg of this substance (approximately 1/2.5 M.L.D.) (Fig. 11.) became remarkable at the 12th hour and a relatively larger number of metaphase, which was seen in about 30%–40% of the tumor cells, was taken over by the scattered diplochromosomes. The above changes lessened after 24 hours, and accumulation of normal metaphase, distorted star metaphase and necrobiotic metaphase appeared mixed with remark-

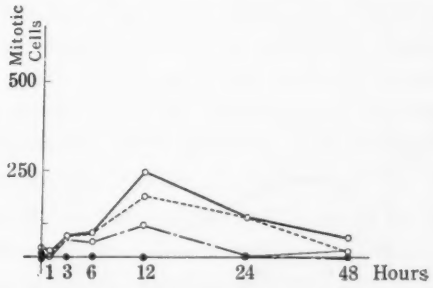
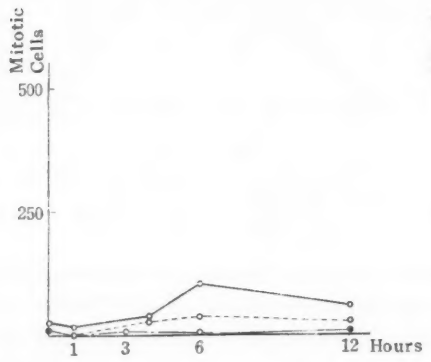


Fig. 12. VI, b.
Thiocolchicine 0.1 mg/100 g subcutaneous injection.

ably destroyed and necrotic tumor cells. After 48 hours, abnormal division of tumor cells was hardly seen. The overall picture caused by subcutaneous injection of 0.1 mg of this substance (approximately 1/5 M.L.D.) (Fig. 12.) showed lesser changes than that of 0.2 mg. It, however, followed almost the same course, and 12 hours later, scattered diplochromosomes accumulated in a rather large number (Plate I, Fig. 1). The distorted picture which was seen at the 24th hour started to recover with the appearance of ana- and telophase at the 48 th hour. After subcutaneous injection of 0.05 mg (approximately 1/10 M.L.D.) (Fig. 13.), scattered diplochromosomes appeared 3-6 hours later. The accumulation of metaphase which appeared at the 6 th hour mostly consisted of normal metaphase and



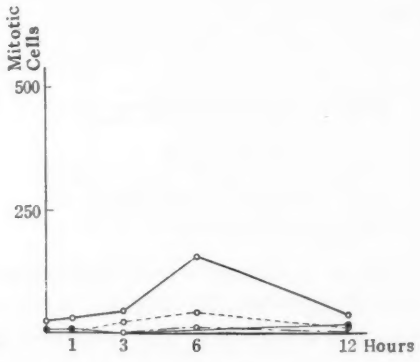


Fig. 13. VI, c.

Thiocolchicine 0.05 mg / 100 g subcutaneous injection.

Thiocolchicine 0.05 mg/100 g subcutaneous injection.

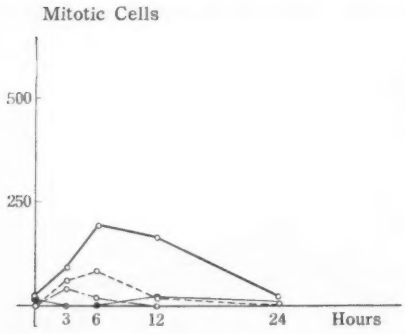


Fig. 14. VI, d.

Thiocolchicine 0.01 mg / 100 g intraperitoneal injection.

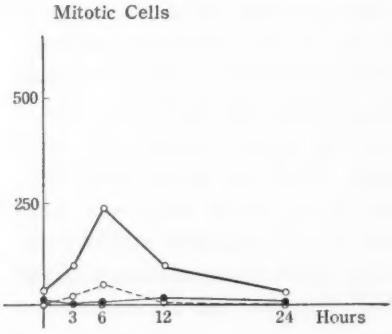


Fig. 15. VI, e.

Thiocolchicine 0.005 mg/100 g intraperitoneal injection.

destorted star metaphase, which showed a tendency to return to normal with the appearance of telophase at the 12 th hour.

An experiment with intraperitoneal injection of thiocolchicine was carried out. Mitoses were at least effectively arrested with 0.01 mg (approximately 1/50 M.L.D.) (Fig. 14.), and scattered diplochromosomes were observed up to 6 hours with this amount. Ana- and telophase showed both signs of recovery at the 6 th hour. Accumulation of metaphase was shown in relatively large number with 0.005 mg (approximately 1/100 M.L.D.) (Fig. 15.), while scattered diplochromosomes were seen only in a small number at the 3 rd hour. Complete disappearance of ana- and telophase was, however, not observed. With 0.002 mg (approximately 1/250 M.L.D.) (Fig. 16.), abnormal mitoses were hardly seen, of which 1 of 2 cases showed slight accumulation of normal mitoses at the 3 rd hour.

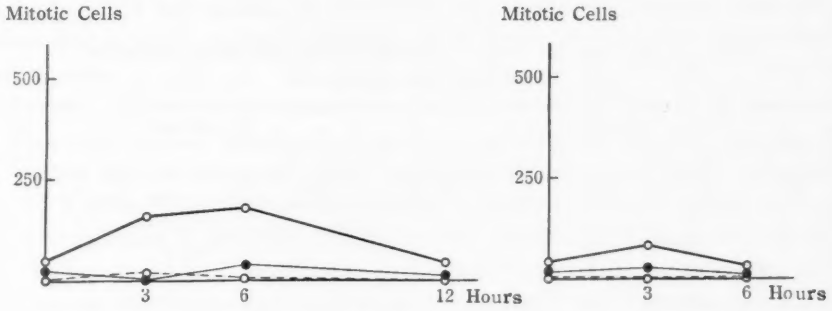


Fig. 16. VI, f.

Thiocolchicine 0.002 mg/100 g intraperitoneal injection.

Thiocolchicine 0.002 mg/100 g intraperitoneal injetion.

4) Thiocolchiceine (VII).[20)21)]

Colchicine-like mitoses appeared an hour after subcutaneous injection of 1 mg of this substance (approximately 1/2 M.L.D.) (Fig. 17.). Abnormal metaphase accumulated in about 20 % of the tumor cells after 6 hours, and these changes were mostly taken over by scattered diplochromosomes. 12 hours later both normal and abnormal metaphases took over half of tumor cells, and the increase destorted star metaphase and ball metaphase became remarkable. 24 hours latter, with the appearance of ana- and telophase, the findings of ascites returned to almost normal. Subcutaneous injection of 0.5 mg (approximately 1/4 M. L. D.) hardly showed any effect on the findings of ascites.

5) Condensation product of colchiceine-hydrazid with acetone (VIII).[20)21)22)]

In M.L.D. test (Table 2), 4 of 5 rats survived with subcutaneous injection of 2 mg, while all died with that of 3 mg. However, the rats which survived the

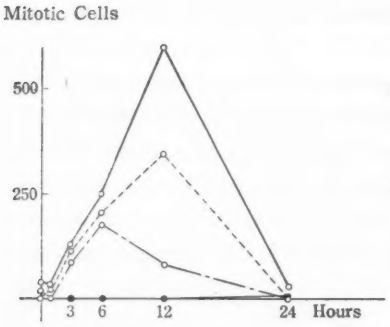


Fig. 17. VII, a.
Thiocolchiceine 1 mg/100 g subcutaneous injection.

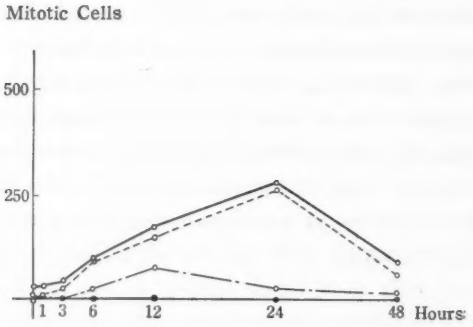


Fig. 18. VIII, a.
Condensation product of colchiceine-hydrazid with acetone 2 mg/100 g subcutaneous injection.

Table 2. The Test for M.L.D. of Condensation Product of Colchiceine-hydrazid with Acetone.

	First test			Second test *		
No.	Body weight (gm.)	Inj. (mg.)	Ist 2nd 3rd 4th day day day day	Body weight (gm.)	Inj. (mg.)	Ist 2nd 3rd 4th day day day day
1	130	3	†			
2	140	3	†			
3	130	3	†			
4	136	3	†			
5	92	2	survival	132	1	survival
6	115	2	survival	122	1	survival
7	125	2	survival	118	2	†
8	115	2	†			
9	135	1	survival	153	2	†
10	140	1	survival	142	1	survival
11	140	1	survival	166	1	survival
12	130	1	survival	140	2	†
13	102	**	survival	108	2	survival
14	100	2	survival	110	—	survival

Indications :

* ...After 45 days from the first test for M.L.D.

** ...3-(p-tolyl sulfonyl) aminotropolone 10mg. subcutaneous injection.

injection of 2 mg and 1 mg, died in toxic conditions with the second test of 2 mg which was administered at 45 days after the first test, while all survived that of 1 mg. On the other hand the rat survived by the injection of 2 mg at 45 days after

the test for M.L.D. of 3-(p-tolyl-sulfonyl) aminotropolone (XVIII). Judging from the above data, M.L.D. should be decided at 3 mg, but the problem still waits to be solved.

With subcutaneous injection of 2 mg (approximately 2/3 M.L.D.) (Fig. 18.) of this substance, abnormal metaphase was observed at the 1st hour, with only slight decrease of ana- and telophase. At the 3rd hour, destorted star metaphase and necrobiotic metaphase appeared. Scattered diplochromosomes appeared at the 6th hour and was at height after 12 hours. While this scattering remained 24-48 hours, the viscosity of chromosomes increased, resulting in strong tendency of coagulation. Accumulation of metaphase was seen at the height at the 24th hour, and both caryolysis and caryorexis of tumor cells became remarkable at the 48th hour, many giant tumor cells being observed. Ana- and telophase showed tendency to decrease after one hour, and disappeared completely 6 hours later. Normal metaphase did not accumulate throughout the experiment. In this study, 3 of 8 cases died in toxic conditions between 24-48 hours, and 5 other cases showed prolongation of survival. The mean survival of 4, except 1 which died on the 118th day, was 13.7 days, while the mean survival of control was 8.7 days.

With subcutaneous injection of 1 mg (approximately 1/3 M.L.D.) (Fig. 19.), the effect was seen at the height at the 12th hour, followed with recovery at the 24th hour. Scattered diplochromosomes started to appear from the 3rd hour and with the accumulation of abnormal metaphase reached the maximum at the 12th hour, but these changes were hardly seen at the 24th hour. Ana- and telophase which disappeared till 12 hour recovered at the 24th hours. Resting tumor cells showed caryorexis and caryolysis after 24 hours. The survival was, however, not prolonged very much with subcutaneous injection of 1 mg. With subcutaneous injection of 0.5 mg (approximately 1/6 M.L.D.) (Fig. 20), accumulation of metaphase was

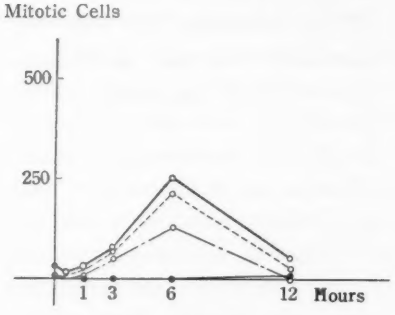


Fig. 19. VIII, b.
Condensation product of colchiceine-hydrazid with acetone 1 mg/100 g subcutaneous injection.

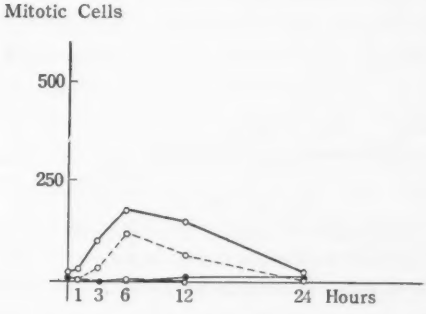


Fig. 20. VIII, c.
Condensation product of colchiceine-hydrazid with acetone 0.5 mg/100 g subcutaneous injection.

slightly observed till the 12th hour, and most of abnormal metaphases showed distorted star metaphase. Scattered diplochromosomes were seen slightly at the 6th hour. Ana- and telophase almost disappeared between 3 and 6 hours, and the visible ones were of abnormal type, without ability to complete mitoses. However, normal ana- and telophase recovered completely at the 12th hour.

6) Condensation product of colchiceine-hydrazid with methyl ethyl ketone (IX).[20]

With subcutaneous injection of 5 mg (approximately M.L.D.) (Fig. 21), 2 mg (Fig. 22) and 1 mg of this substance (Fig. 23), slight accumulation of abnormal metaphase

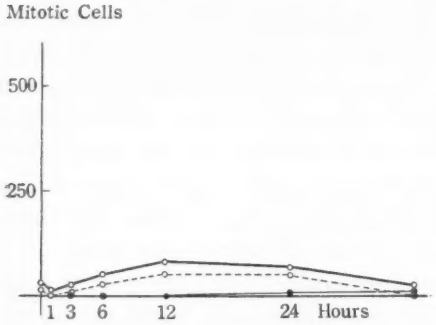


Fig. 21. IX, a.
Condensation product of colchiceine-hydrazid with methyl ethyl ketone 5 mg/100 g subcutaneous injection.

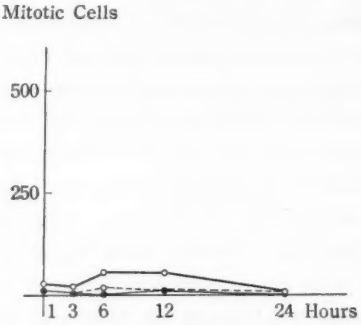
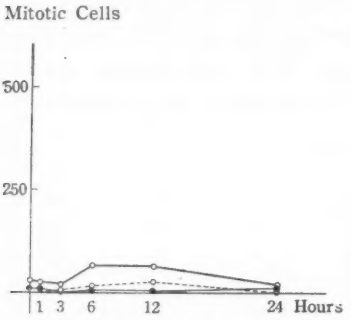


Fig. 22. IX, b.
Condensation product of colchiceine-hydrazid with methyl ethyl ketone 2 mg/100 g subcutaneous injection.



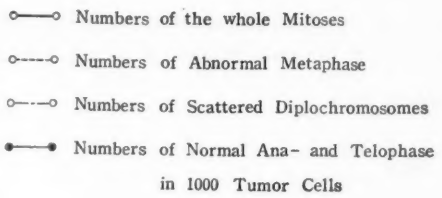


Fig. 23. IX, c.
Condensation product of colchiciene-hydrazid with methyl ethyl ketone 1 mg/100 g subcutaneous injection.

without complete disappearance of polarity, such as star metaphase and necrobiotic star-like metaphase, appeared in small number. Ana- and telophase did not disappear completely with less than 5 mg, but with 5 mg, ana- and telophase only

disappeared between 3–6 hours. With 5 mg, its influence was observed till the 24 th hour, while only till the 12 th hour with 2 mg or 1 mg.

7) Colchiceinamide (X).[11)14)]

Accumulation of abnormal metaphase reached the maximum at the 12 th hour after subcutaneous injection of 1 mg of this substance (approximately 1/2 M.L.D.) (Fig. 24), and scattered diplochromosomes were observed remarkably at this time. However, these changes could be hardly observed at the 24 th hour and cell division returned to normal. The extracellular outflow of cytoplasma was observed 1–3 hours after injection remarkably. With subcutaneous injection of 0.5 mg (approximately 1/4 M.L.D.) (Fig. 25), scattered diplochromosomes which appeared

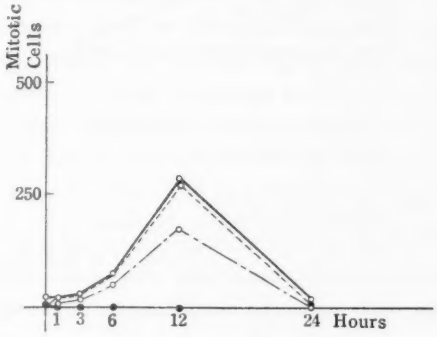


Fig. 24. X, a.
Colchiceinamide 1 mg/100 g subcutaneous injection.

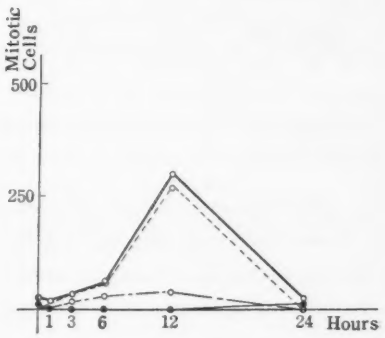


Fig. 25. X, b.
Colchiceinamide 0.5mg/100g subcutaneous injection.

at the 1 st hour did not accumulate very much through the entire course. At the 12 th hour star metaphase and necrobiotic star-like metaphase were remarkably accumulated. Ana- and telophase which disappeared up to 6 hours after injection began to appear at the 12 th hour, although all of them showed abnormality. At the 24th hour the whole picture returned to normal. With subcutaneous injection of 0.2 mg (approximately 1/10 M.L.D.) (Fig. 26), a slight accumulation of metaphases, which were chiefly of star metaphases, and a formation of abnormal ana- and telophase were observed till the 6 th hour. The whole picture, however, showed complete recovery to normal at the 12 th hour. The extracellular outflow of cytoplasma was remarkably seen one hour after injection of 0.5 mg and 0.2 mg. A slight accumulation of metaphase, which was mostly star metaphase, was observed up to 6 hours after subcutaneous injection of 0.1 mg (approximately 1/20 M.L.D.) (Fig. 27), while ana- and telophase appeared mingled with a few polypolar formations at the 6 th hour. 12 hours later the whole picture recovered to normal.

Few changes were observed one hour after subcutaneous injection of 0.05 mg (approximately 1/40 M.L.D.).

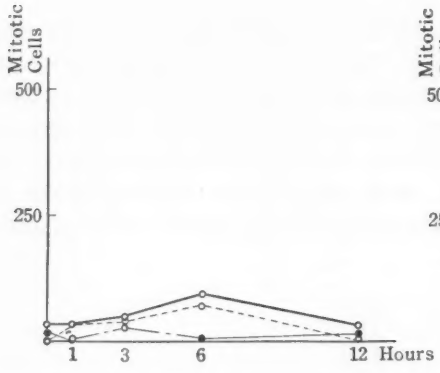


Fig. 26. X, c.
Colchiceinamide 0.2 mg/100 g subcutaneous injection.

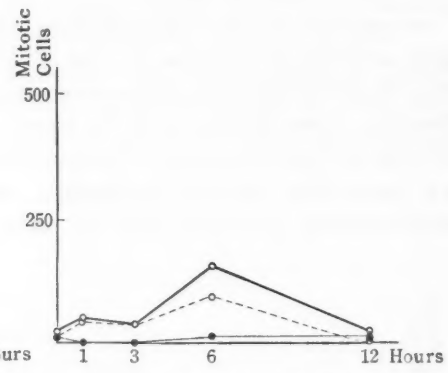


Fig. 27. X, d.
Colchiceinamide 0.1 mg/100 g subcutaneus injection.

8) Condensation product of colchine with guanidine (XI).[20]

3 hours after subcutaneous injection of 10 mg of this substance, a slight degree of accumulation of normal metaphase and also star metaphase was seen. With subcutaneous injection of 20 mg (Fig. 28), there appeared accumulation of normal

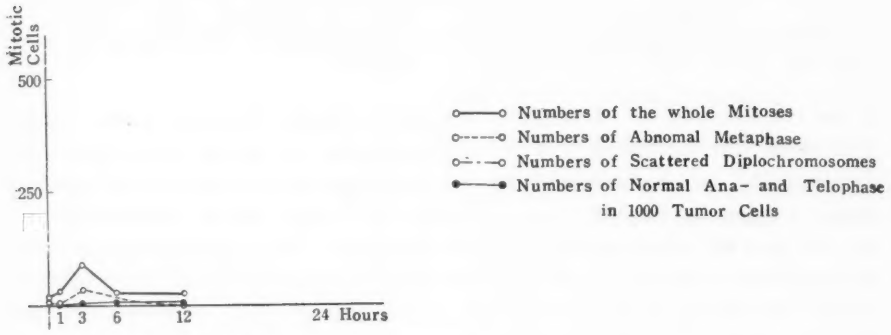


Fig. 28. XI, a.
Condensation product of colchicine with guanidine 20 mg/100 g subcutaneous injection.

metaphase with slight increase of star metaphase and a few scattered diplochromosomes. With subcutaneous injection of 30 mg and 40 mg, slight changes were seen as with 20 mg, while both of them died 24-48 hours after injection.

Add. 1. S-ethyl thiocolchiceine (XII).[20] Due to the shortage of the material, subcutaneous injection above 2 mg could not be studied. The result observed with subcutaneous injection of 2 mg. and lesser dosage did not show any influence.

Add. 2. 5-Amino-4 (β-hydroxy-γ-phenyl-propyl) tropolone triacetate (XIII).[23] Effect of arresting mitoses was not observed with this substance. It has, however, interesting muscle relaxing effect.

Hindleg paralysis occurred 10 minutes after 30 mg was injected into the abdominal cavity of healthy rats, and complete paraplegia was observed 15 minutes later. Paralysis of the forelegs recovered at 45 minutes, with remaining paralysis of the hind-legs which recovered an hour later. The rats ran around again. No respiratory paralysis was observed. 2 of 4 died on the 2 nd and 4 th day. With intraperitoneal injection of 40 mg paralysis of both legs occurred 10 minutes later, and complete loss of movement was observed between 15 and 30 minutes. The paralysis of the forelegs recovered an hour later, while the hind-legs returned to normal an hour and half, or 2 hours later. Respiratory paralysis was not seen. 2 cases which were used for the test died on the 2 nd and 4 th day, respectively. The above mentioned state was hardly seen in cases of intraperitoneal administration of 5-amino-4 (β-hydroxy-γ-phenyl-propyl) tropolone (XIV).[24]

It is interesting to notice that this fact coincides with the reports that colchicine is a poison to nerve.

Discussion

1) In evaluating the effect on mitotic activity of colchicine derivatives morphological changes were chiefly discussed with little consideration on the duration of effect. However, it is doubtful whether a short-acting substance produces the suppressive effect on division of tumor cells or not.

Unquestionably, the strength of action depends on the time factor. If the minimum dose to induce accumulation of abnormal metaphase and disappearance of ana- and telophase, without consideration on the duration of effect, is decided as minimum effective dose, the following 6 substances have effective dose lesser than M.L.D., i.e., colchicine, T.M.C.A. methyl ether d-tartrate, thiocolchicine, thiocolchiceine, condensation product of colchiceine-hydrazid with aceton and colchiceinamide as reported in this paper. The M.L.D./M.E.D. is 2, 100, 10, 2, 6 and 20, respectively. If the mimum dose which induces abnormal metaphase accumulation and ana- and telophase disappearance up to 12 hours is decided as M.E.D. 12 hours, the M.L.D./M.E.D. 12 hours of the above 6 substances are 2, 10, 5, 2, 3 and 4, respectively (Table 3). If the above effect is considered up to 24 hours, there are 3 substances, i.e., T.M.C.A. methyl ether d-tartrate, thiocolchicine and condensation product of colchiceine-hydrazid with acetone, and their M.L.D./

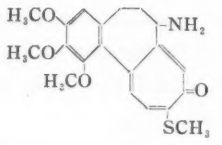
Table 3.

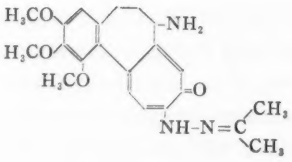
	M.L.D. / M.E.D. 3hrs.	M.L.D. / M.E.D. 6hrs.	M.L.D. / M.E.D.12hrs.	M.L.D. / M.E.D.24hrs.	M.L.D. / M.E.D. 48hrs.
Colchicine	2	2	2	—	—
T.M.C.A. methyl ether d-tartrate	50	25	10	2.5	—
Thiocolchiceine	10	10	5	5	—
Thiocolchicine	2	2	2	—	—
Condensation product of colchiceine-hydrazid with acetone	6	6	3	1.5	1.5
Condensation product of colchiceine-hydrazid with methyl ethyl ketone	1	1	1	—	—
Colchiceinamide	20	10	4	—	—

M.E.D. 24 hours are 2.5, 5 and 1.5, respectively.

2) From the above experiment, when the side chain of B and C ring of colchicine are studied in details, in B ring, $-NH_2$ is superior to $-NHCOCH_3$ in strength, and in C ring, the strength follows $-SCH_3$, $-NH-N=C\begin{matrix}CH_3\\CH_3\end{matrix}$, $-OCH_3$ and $-SH$ in the order given.

Therefore, colchicine derivatives with the following structure seem to have an interesting effect.





Experiment (2)

Repeated Administration with Colchicine Derivatives

Prolongation of survival period, and cytological changes were studied by repeated administration of colchicine derivatives. The amount of administration was selected enough to suppress mitoses. Side-effects during administration was controlled fairly well by subcutaneous injection of 2 cc of physiologic saline solution per 100 g body weight a day. The body weight of rat was average 100–120 g, with exceptional use of 120–150 g.

1) Solitary Continuous Administration of Trimethylcolchicinic Acid Methyl Ether d-tartrate.

With continuous administration of this substance, first injection of 0.5 mg followed

with 0.2 mg every 12 hour was found to be the most effective method. When the total dosage by continuous administration was over 2 mg per 100 g body weight, many died in toxic conditions. With continuous administration 4 days after transplantation, accumulated metaphase, especially, the abnormal metaphase reached its maximum 48 hours after the beginning of injection, when total number of metaphase occupied about 40% of tumor cells and most part of them showed abnormal findings. Scattered diplochromosomes reached its maximum 24 hours after injection, and at the 48th hour star metaphase, ball metaphase and necrobiotic metaphase were chiefly found. The accumulated metaphase remarkably decreased 60 hours after injection, accompanied with conspicuous decrease of numbers of tumor cells in 1 mm^3. Telophase was, however, observed in a small number at this time. It was impossible to wipe them out by further administration. The destruction of resting cells was observed 12-24 hours in a form of extracellular outflow of cytoplasma. The appearance of giant cells was outstanding from the 4 th day. Ana- and telophase returned nearly to normal 4 days after the injection of substance. The survival period was prolonged in comparison with that of control.

2) Solitary Intermittently Repeated Administration of Thiocolchicine.

In cases of continuous administration of this substance, it was found most effective to inject 0.1-0.05 mg every 12 hour. When the dosage of continuous administration reached 0.5 mg in total, many died in toxic conditions. With continuous subcutaneous administration of thiocolchicine at the 4 th day after transplantation, the same findings as in the case of T.M.C.A. methyl ether d-tartrate were observed. The accumulation of metaphase to reach its maximum was delayed, which was 60 hours after injection, while scattered diplochromosomes were found at most at the 48 th hour. Necrobiotic and necrotic pictures of resting cells became remarkable at the 48 th hour, fairly large number of giant cells appeared from the 72 nd hour, which was accompanied with active phagocytosis by many inflammatory cells, such as leucocyte, monocyte, etc.. The survival period prolonged as much as with trimethylcolchicinic acid methyl ether d-tartrate, but less infiltration of tumor cells was found at autopsy. In case of intraperitoneal continuous administration at the 4 th day after transplantation, the survival period could be prolonged more than by subcutaneous injection. This tendency was found more conspicuous in rats 4-12 hours after transplantation than in those 4 days after transplantation. The method of administration was studied on rats 4 hours after transplantation with the results shown in Fig. 29. The first and second groups survived longer than the control, but they survived lesser than the third group, which had 0.1 mg 4 times for the earlier administration followed with enough supplemental administration. The picture of destruction of cell division

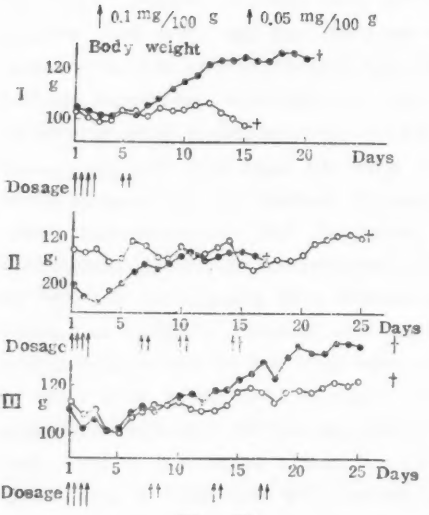


Fig. 29.

Solitary Intermittently repeated Administration of Thiocolchicine intraperitoneal injection 4 hours after transplantation.

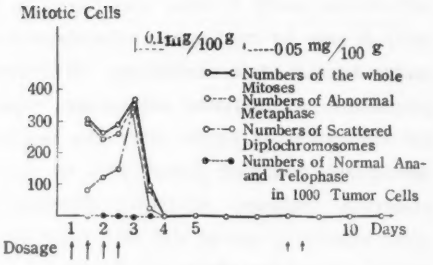


Fig. 30.

Solitary Intermittently repeated Administration of Thiocolchicine intraperitoneal injection 4 hours after transplantation.

is shown in Fig. 30. However, in every group, considerable decrease of body weight was noticed by administration in the earlier period.

3) Alternating and Combined Use of Colchicine Derivatives.

Four colchicine derivatives, i.e., T.M.C.A. methyl ether d-tartrate, thiocolchicine, colchiceinamide and condensation product of Colchiceine-hydrazid with acetone, were injected alternatingly and continuously into the abdominal cavity as shown in Table 4 and Fig. 31, 4-12 hours after transplantation. By such alternating combined method, rats survived about 20 days after transplantation. The process of destruction of cell division is shown in Fig. 32. After starting administration, metaphase gradually started to accu-

Table 4.

Ⓐ	Trimethylcolchicinic acid methyl ether d-tartrate 0.5mg/100g (1/10 M.L.D.)
Ⓐ	Trimethylcolchicinic acid methyl ether d-tartrate 0,2mg. (1/25 M.L.D.)
Ⓑ	Thiocolchicine 0.05mg. (1/10 M.L.D.)
Ⓑ	Thiocolchicine 0.02mg. (1/25 M.L.D.)
Ⓒ	Colchiceinamide 0.1mg. (1/20 M.L.D.)
Ⓓ	Condensation Product of Colchiceine-hydrazid with acetone 0.2mg. (1/15 M.L.D.)

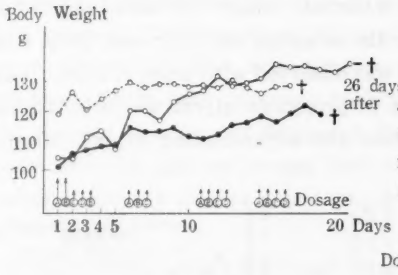


Fig. 31.
Alternative and Combined Use of 4 Colchicine Derivatives intraperitoneal injection 12 hours after transplantation.

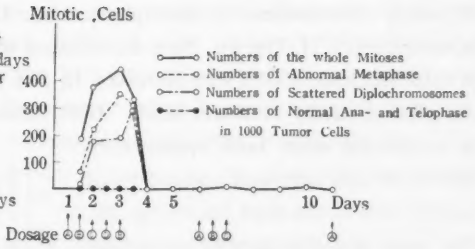


Fig. 32.
Alternative and Combined Use of 4 Colchicine Derivatives intraperitoneal injection 12 hours after transplantation.

mulate, and took over about 40% of tumor cells on the 3rd day, which was accompanied with abnormal metaphase, especially with scattered diplochromosomes. Thereafter, cell division decreased and it looked as if it disappeared. Several normal mitoses appeared at around 20 days. With this alternating and combined administration of colchicine derivatives, the body weight of rats did not decrease during the whole course.

Discussion

The effect of colchicine derivatives to prolong life is still unsatisfactory. Solitary intermittent administration of thiocolchicine and alternating combined administration of 4 colchicine derivatives in a earlier period after transplantation showed demonstrable prolongation of life.

In case of intermittent repeated administration of thiocolchicine, decrease of body weight was seen in the earlier period of administration, while intermittent administration of 4 derivatives did not show decrease of body weight. It may be better to use the latter than the former in case of human application.

Experiment (3)

Clinical Application of Colchicine Derivatives

The picture of tumor cells in two clinical cases with cancerous pleurisy was studied with alternating combined use of colchicine derivatives, which was tested in animal experiments.

Case 1. Diagnosis. Pleuritis carcinomatosa dextra. 56 years old woman. After middle lobe lobectomy was carried out for primary cancer of the right lung which was confined to segment 4 (Plate I, Figs. 2 and 3), many tumor cells were demonstrated in the punctate of right pleural cavity on the 11 th postoperative day (Plate, Fig. 4). Mitoses were found in nearly 2% of tumor cells. With intrapleural

injection of 20 mg T.M.C.A. methyl ethyl d-tartrate which was dissolved in 5 cc of water, chromosomes in metaphase made the so-called ski pair and they were in disorder (Pl. II, Fig. 5). Its accumulation was observed with lapse of time. 10 mg of thiocolchicine which was dissolved in 2 cc of propylene glycol was injected into the pleural cavity 24 hours later. Destruction and accumulation of cell division in metaphase were both remarkably shown, which occupied about 20% of tumor cells at the 33 rd hour (Fig. 33). The type of destruction of metaphase which appeared during this time was chiefly scattered diplochromosomes (Pl. II, Fig. 6), distorted star metaphase, ball metaphase, coagulation and necrobiotic metaphase. Ana- and telophase completely disappeared after injection. Though the changes of resting cells were silght, giant cells appeared. After administration of thiocolchicine, migration of leucocytes increased and phagocytosis of tumor cells was remarkable. 48 hours later, tumor cells completely disappeared (Table 5). Slight migration of leucocytes and monocytes together with fibrin was seen

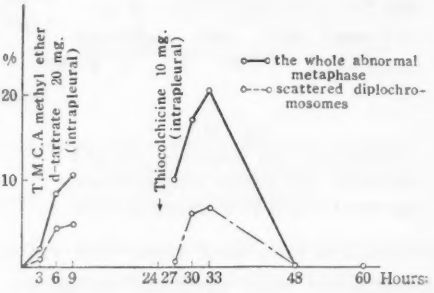


Fig. 33.

Case 1. Pleuritis carcinomatosa. (56 years old woman) Incidence of arrested metaphase in tumor cells.

Table 5. Pleuritis carcinomatosa 56 yrs. female pleural effusion

	counted numbers of tumor cells	numbers of normal pro- and metaphase	numbers of abnormal metaphase	numbers of normal ana- and telophase
before injection	1000	4	0	15
injection after	Trimethyl colchicinic acid methyl ether d-tartrate 20mg. intrapleural			
3 hrs.	500	1	10	0
6 hrs.	500	0	46	0
9 hrs.	250	0	27	0
24 hrs.	Thiocolchicine 10mg. intrapleural injection			
27 hrs.	40	0	4	0
30 hrs.	150	0	23	0
33 hrs.	88	0	20	0
48 hrs.	0	0	0	0
6 days	0	0	0	0
7 days	0	0	0	0
10 days	0	0	0	0
15 days	Pleural effusion is not obtained.			

in pleural punctate on the 6 th, 7 th and 10 th day. It became impossible to obtain punctate 15 days after injection. Patient complained of severe chest pain when pleural injection was performed, followed with transient accumulation of exudate. Chest pain appeared from the 3 rd hour after injection, increased with the severity of action of drug, and disappeared when it was impossible to remove punctate. The patient became free of complaint and was discharged from the hospital about 2 months later. After about one year, she died with recurrence of the disease.

Case 2. Diagnosis. Malignant struma with metastasis to the left lung complicated with carcinomatous pleurisy. 63 years old man. The x-ray picture (Pl. II, Fig. 7) showed remarkable accumulation of pleural exudation. Pleural punctate (1,800 cc) disclosed many tumor cells (Pl. II, Fig. 8). 3 mg thiocolchicine dissolved in propylene glycol was injected into the pleural cavity and 2 mg dissolved in 1 cc of propylene glycol which was added to physiologic saline solution was injected by the intravenous route by drops. 6 mg thiocolchicine was injected 4 times intravenously by drops twice every other day, followed with 4 mg colchicine 2 days later by the same method of administration. However, severe diarrhoea continued for 10 days, and the injection was suspended temporarily. Later on 10 mg T. M. C. A. methyl ether d-tartrate followed with 20 mg of the substance 6 days later was administered. The following day 10 mg thiocolchicine was given and 2 days later, followed with 10 mg colchiceinamide and 2 days later, followed with 10 mg condensation product of colchiceine-hydrazid with acetone which caused leucopenia. Consequently, the administration had to be suspended temporarily. With such method of administration, tumor cells decreased and were replaced with fibrin formation (Pl. II, Fig. 9). With these changes of punctate, the x-ray shadows of lung diminished and the pleural punctate could not be removed (Pl. II, Fig. 10). Preexsiting chest pain, cough and dyspnea were greatly ameliorated. Leucopenia recovered within 10 days. Treatment was resumed one month later by administering 80 mg T.M.C.A. methyl ether d-tartrate, 27 mg thiocolchicine, 32 mg colchiceinamide and 10 mg condensation product of colchiceine-hydrazid with acetone. However, cancer cells resistant to drug gradually appeared with recurrence of symtoms and the patient died 6 months later. At autopsy, pleura was remarkably hypertrophied, and 500 cc pleural exudate accumulated. Tumors on bilateral neck were not greatly influenced.

Discussion

According to Lits[25], the first application of colchicine on human cancer was made by Dominici[26] followed with reports by Oughterson[27], von Brücke[28], Seed[29], and others. The effect, though favorable, was far from being satisfac-

tord. Reports of colchicine derivatives on human neoplasm were published by Moeschlin[30], Bock[31], Storti[32] and Akaishi et al.,[22] the former investigators using demecolcine.

According to the data by Moeschlin[30], demecolcine can well be recommended for continual oral therapy of chronic myeloic leukemia.

Of 2 clinical cases herein reported, case I was early postoperative occurrence of carcinomatous pleurisy, in which mitoses occupied 2% of tumor cells. It showed mitoses in active stage. Remarkable destruction of cell division was observed by direct intrapleural injection. Case 2 was in a rather late stage of the disease with few mitoses of tumor cells. Therefore, it took time to destroy tumor cells with little influence on pulmonary metastasis. The pleural effusion was, however, transiently held down. From the above data, in case of applying colchicine derivatives, it seems that the more tumor cells in wandering and active mitoses the more the destruction of tumor cells. It is, however, impossible to expect complete chemotherapy of human cancer with present colchicine derivatives.

Summary and Conclusion

1) Study was made on suppressive effect on cell division with colchicine and tropolone derivatives, of which four i. e., T. M. C. A. methyl ether d-tartrate, thiocolchicine, colchiceinamide and condensation product of colchiceine-hydrazid with acetone had stronger effect than colchicine. These involved different time factors to show their effect. In order to decide the degree of strength, it was found that study on M. L. D./M. E. D. was not enough, but necessary to compare M. L. D./M. E D. 12 hours with M. L. D./M. E. D. 24 hours. In conclusion, it was suggested that in the derivatives of colchicine the sulfur molecule and -NH-N $=C\langle^{CH_3}_{CH_3}$ on the C-ring of colchicine play a comparatively important role.

2) With colchicine derivatives it seems favorable with less toxicity, to use combined method of administration than its solitary use to destory tumor cells of rats transplanted with Yoshida's sarcoma.

3) Cytological changes were observed in studying the application of colchicine derivatives on 2 clinical cases. When tumor cells were in wandering and had many mitoses, the division of tumor cells were suspended and destroyed in metaphase. However, it is impossible to expect complete chemotherapy of human cancer with present colchicine derivatives. Further investigation are needed on new derivatives and chemical agents which destroy resting tumor cells and on the use of combined administration.

References

1) Pernice, B.: Sicilia Med., **1**, 265 (1889). Cited from Eigsti, O. J., et al.: Science, **110**, 692 (1949).

2) Amoroso, E. C.: Nature, London, **135**, 266 (1935).

3) Lits, F. J.: Compt. rend Soc. de biol., **115**, 1421 (1934).

4) Brues, A. M., and Cohen, A.: Biochemical J., **30**, 1363 (1936).

5) Dewar, M. J. S.: Nature, London, **155**, 141 (1945).

6) Windaus, A.: Annalen, **439**, 59 (1924). Cited from Dewar, M. J. S.[5]

7) Nozoe, T., Seto, S., Kitahara, Y., Kunori, M., and Nakayama, Y.: Proc. Japan Acad., **26**, 38 (1950).

8) Doering, W. E., and Knox, L. H.: J. A. C. S., **72**, 2305 (1950).

9) Cook, J. W., Gibb, A. R., Raphael, R. A., and Somerville, A. R.: Chem. Ind., 427 (1950).

10) Haworth, R. D., and Hobson, J. D.: Chem. Ind., 441 (1950).

11) Malinsky, J., and Santavy, F.: Lek. listy, **11**, 276 (1952).

12) Goldberg, B., Ortega, L. G., Goldin, A., Ullyot, G. E., and Schoenbach, E. B.: Cancer, **3**, 124 (1950).

13) Leiter, J., Downing, V., Hartwell, J. L., and Shear, M. J.: J. Nat. Cancer Inst., **13**, 379 (1952).

14) Leiter, J., Hartwell, J. L., Kline, I., Nadkarni, M. V., and Shear, M. J.: J. Nat. Cancer Inst., **13**, 731 (1952).

15) Katsura, S-T., Sato, K., Akaishi, K., Nozoe, T., Seto, S., and Kitahara, Y.: Proc. Japan Acad., **27**, 31 (1951).

16) Katsura, S-T., Sato, K., Akaishi, K., Nozoe, T., Seto, S., and Ebine, S.: Proc. Japan Acad., **27**, 36 (1951).

17) Katsura, S-T., Sato, K., Akaishi, K., Nozoe, T., and Kitahara, Y.: Proc. Japan Acad., **27**, 250 (1951).

18) Sato, K.: Tohoku Ishi. (in Japanese), **49**, 18 (1954).

19) Sato, H.: Gann, **39**, 112 (1948).

20) Nozoe, T., Ikemi, T., and Ito, S.: Sci. Rep. Tohoku Univ. Ser. I., **38**, 117 (1954).

21) Akaishi, K., Chiba, K., and Sato, K.: Gann, **45**, 507 (1954).

22) Akaishi, K., Yoshimatsu, S., Akama, Y., and Sato, K.: Gann, **46**, 498 (1955).

23) Nozoe, T., Kitahara, Y., and Doi, K.: to be published.

24) Nozoe, T., Kitahara, Y., and Doi, K.: Proc. Japan Acad., **29**, 203 (1953).

25) Lits, F. J.: Arch. internat. de méd. expér., **11**, 811 (1936).

26) Domonici, A., et al.: Thérapeutique Médicale. V. Peau, Syphilis, Cancer. Masson et Cie, Paris (1932).

27) Oughterson, A. W., Tennant, R., and Hirshfeld, J. W.: Proc. Soc. Exper. Biol. and Med., **36**, 661 (1937).

28) von Brücke, E. T., and von Hueber, E. F.: Klin. Wchnschr., **18**, 1160 (1939).

29) Seed, L., Slaughter, D. P., and Limarzi, L. R.: Surgery, **7**, 696 (1940).

30) Moeschlin, S., Meyer, H., and Lichtman, A.: Schweiz. Med. Wschr., **83**, 990 (1953).

31) Bock, H. E., and Gross, R.: Acta Haemat., **11**, 280 (1954).

32) Storti, E., and Gallinelli, R.: Schweiz. Med. Wschr., **84**, 612 (1954).

要　旨

不飽和7員環状化合物，特に Colchicine 誘導体の制癌作用に関する研究

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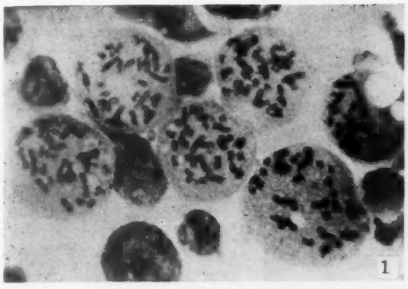
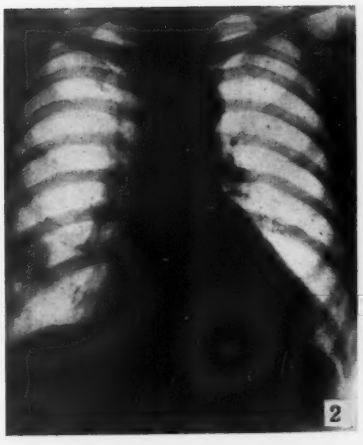
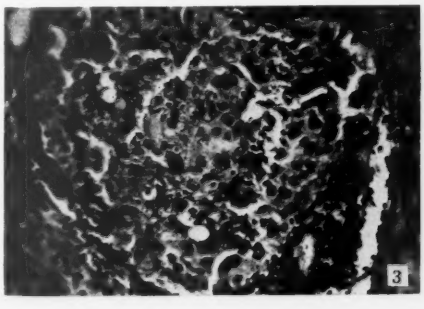
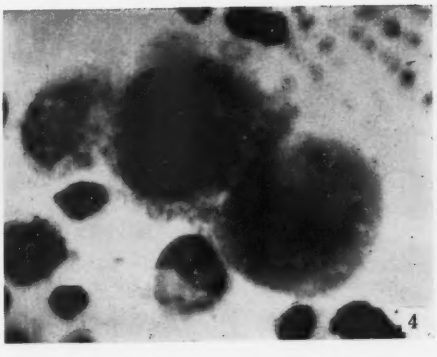
Colchicine 誘導体を主とした20種の不飽和7員環状化合物を吉田肉腫に試みた。Screening test で colchicine より効果のあったのは T. M. C. A. methyl ether d-tartrate, thiocolchicine, colchiceinamide 及び condensation product of colchiceine-hydrazid with acetone の4種であった。これらはそれぞれ作用の持続時間が異なるので，これを詳細に検討した。作用強度を表わすためには $\frac{\text{M.L.D.}}{\text{M.E.D.}}$ のみでは不充分であり，$\frac{\text{M.L.D.}}{\text{M.E.D. 12 hrs.}}$ 及び $\frac{\text{M.L.D.}}{\text{M.E.D. 24 hrs.}}$ を比較することが必要であった。この結果は colchicine の C- 環では硫黄原子及び $-NH-N=C\langle^{CH_3}_{CH_3}$ 基が比較的重要な意義を持つことを示した。また colchicine 誘導体を連続投与する場合は単独で用いるよりも併用した方が毒性少くして同じような効果をあげ得た。これを臨床的に用いた場合には，腫瘍細胞が浮遊状態にあって細胞分裂の盛なものは破壊され易く，腫瘤を形成したものには余り有効ではなかった。

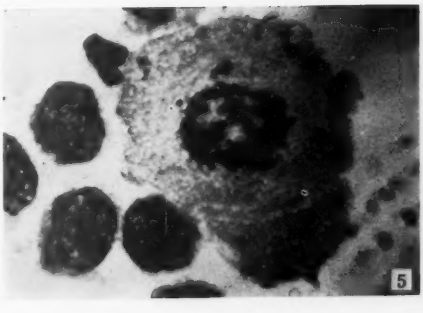


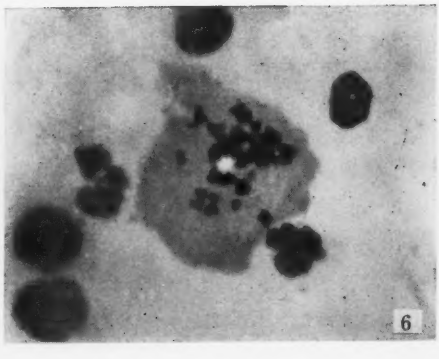
The findings of rat ascites at the 12th hour after snbcutaneous injection of thiocolchicine 0.1 mg.









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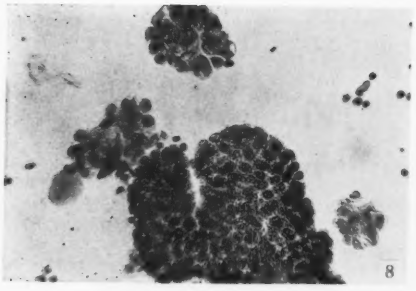
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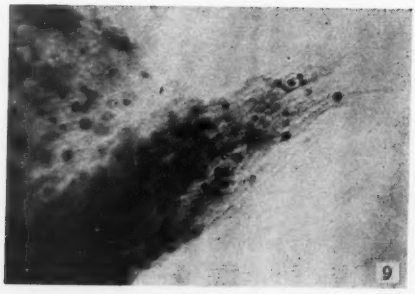
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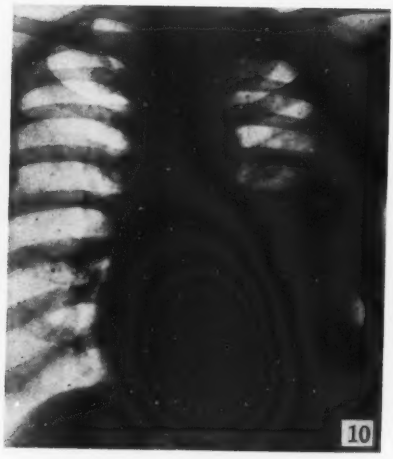
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[GANN, 48, 39~56; March, 1957]

ELECTRON MICROSCOPIC CYTO-HISTOPATHOLOGY (III) ELECTRON MICROSCOPIC STUDIES ON SPONTANEOUS MAMMARY CARCINOMA OF MICE

(With Plates III–IX)

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Since the initial report on the existence of extrachromosomal factor ('33)[1] in the occurrence of mammary cancer in mice, the experimental studies on mammary cancer of mice have been carried out by many researchers in various fields, genetically, pathologically, virologically, biochemically and biophisically through the application of their own methods in experimental cancer research. And from the most of their results, it has highly been suggested that the occurrence of mammary cancer in mice may mostly owe to the transmissible agent which has many of the chracteristics of virus in their mother's milk[2-5]. Thereafter, many attempts have been made to demonstrate the agent with the electron microscope. Graff et al. ('47)[6] reported the presence of a "heavy particle" of virus-like dimensions in the ultracentrifuged milk of high-cancer strain mice. Passey et al. ('47)[7] also found a particulate component in the water extract of desiccated mammary cancer tissue of mice of high-cancer strain, and described that the particulate component was not found in the extract of mammary tissue from low-cancer strain. In 1948 Porter and Thompson[8] studied thinly extended marginal portion of C_3H mouse mammary cancer cells which were cultured on the formvercoated slide glass in vitro with the electron microscope. They found in the preparations taken from three out of six tumors small and unusual particulate bodies, which were associated with the cells and appeared to have spherical shape and double structures consisting of a dense center and less dense outer zone. According to them, the whole particle diameter was about 130 mμ and the dense center was 75 mμ in diameter. Although they observed the cancer cells with the electron microscope, the preparations were too thick to show various internal structures of mammary cancer cells. After the description of Porter and Thompson, electron microscopy of the virus-like particles in situ in mammary cancer cells of mice were done by Bang and Andervont ('53)[9], Dmochowski et al. ('53)[10] and Bernhard et al. ('55)[11] using the advanced ultrathin-sectioning methods. Although these authors observed the virus-like particles, they gave no descriptions about the other cellular structures of mammary cancer cells of mice.

In this paper, electron microscopic observations of ultrathinly sectioned mammary cancer cells of C_3H and DBA strain mice will be reported.

Materials and Methods

The materials for this study were taken from six spontaneous mammary tumors and several metastasized lymphnodes of four C_3H Kinoshita-Takizawa strain mice*, four tumors of two DBA Kinoshita-Mori strain mice* and a lactating mammary gland tissue of a female German Mouse which is considered to have a low incidence of cancer production and had born several babies a week ago. All spontaneous and metastasized mammary tumors were also observed photomicroscopically with routine and several histochemical techniques, and confirmed to be typical, relatively well differentiated adenocarcinomas.

Small tissue blocks of about 1 cubic milimeter were rapidly removed from these individual tissues above described, and fixed in 1 % osmium tetroxide solution adjusted at Ph: 7.40 with phosphate or veronal buffer 2 to 3 hours at room temperature. After fixation, these tissue blocks were washed for 1 hour in destilled water, dehydrated in a series of ethanol, immersed in a monomer mixture consisting of 3 parts of methylmethacrylate, 7 parts of buthylmethacrylate and 20 mg/cc of benzoyl peroxide, and then polymerrized for about 20 hours at 45 degrees centigrade. Thin sections about 30 mμ in thickness were cut with glass knives[12] on the Serval Cantilever Action Microtome[13]. These thin sections cut were floated onto 20% aceton solution, extended on hot water of about 50 degrees centigrade, lifted on collodion coated mesh grids, and desiccated in a vacuum pump.

Observations were done with a JEM-4 C electronmicroscope without removing the embedding plastic purposely by sublimation under the massive electron beam.

Electron microscopy of Cancer cells

1) **Nucleoli.** In this study, only the interphase nuclei and the nucleoli were considered. Cancer cells show commonly two or more nucleoli in a same nucleus. The nucleoli of C_3H and DBA mammary cancer cells are quite large and consist of dense granules and fine twisted thread-like structures made up of linearly arranged very fine granules of moderate density. They also show irregular forms and no limitting membranes. Most of the nucleoli are situated within the nucle-oplasm, but sometimes are attached on the inner surfaces of the nuclear membranes.

At some sites the demarcations between nucleoli and nucleic substance are not

* These six mice are the descendants of two couples of C_3H and DBA strain mice which were imported into Japan from United States by Dr. R. Kinoshita in 1929, and had been bred by Dr. N. Takizawa and Dr. K. Mori. During and after the 2nd World War, these C_3H and DBA strain mice have been kept and bred by U. Takasugi, a laboratory animal breeder, and up to date, both strains of mice have showed almost 95 % incidence of caner production in about 60 generations.

evident, and show gradual transition.

Bernhard et al ('55)[14] studied electron microscopically ultrastructures of nucleoli of normal and cancer cells, and observed the network-like or tangled string-like aggregations of nucleolar substance. The author has also noted such a profile of nucleoli in several human cancer cells (unpublished observations).

And in this study, these figures of nucleoli in cancer cells of the mice could often be observed (Fig. 6, 8), but in some of the normal control cells such figures of nucleoli resembling those seen in cancer cells were also found (Fig. 1), and these may not be a characteristic feature of nucleoli in cancer cells but one suggesting the prominent protein synthetic activity in normal and malignant cells.

2) **Nuclei.** The form of nuclei is usually oval or ellipsoid, but the more atypical forms are very common, furthermore the double nucleated cancer cells are frequently seen. The nncleoplasm in both kinds of cancer cells also consists of fine two kinds of granular materials. One of the two kinds of granules is abundant in number and has a lower density and a poorer demarcation than those of another which is much more few in number and has a relatively high electron-density and relatively clear demarcation, and the former shows fine twisted thread like arrangement which sometimes seem to be circular (Fig. 3, 4, 8, 11).

The distribution of these nucleoplasmic components is usually even throughout most nuclei, but sometimes they show various sizes and forms of denser aggregates attaching to the inner surface of the nuclear membrane (Fig. 6), and some of these may show early prophase of mitosis as observed by Selby ('53)[15]. The nuclear membranes of the carcinoma cells generally show lesser regularity in their outline than those of normal lactating cells of the German Mouse (Fig. 1, 2, 6, 3), but their double nature can clearly be seen even in cancer cells (Fig. 1, 3, 6-8, 11). The abnormal form of nuclei accompanied by irregular shape of protrusions or indentations of nuclear membranes is fairly common, and sometimes deeply inserted canalicular or fissure-like indentations of nuclear membrane into nucleoplasm are also noteable.

3) **Endoplasmic reticulum (Porter), Ergastoplasmic Sac (Weiss) and Intracytoplasmic Sac (Watanabe).** This prominent intra-cytoplasmic structure has long been observed by many investigators such as Porter ('45, '53, '54)[16-18], Dalton ('50, '51)[19,20], Dalton and Striebich ('51)[21], Palade ('52, '53)[22-24], Palade and Porter ('52, '54)[25,26], Watanabe ('53, '55)[27,28], Gautier ('53)[29], Weiss ('53)[30], Rinehart ('53)[31], Sjöstrand ('53)[32], Challice and Lacy ('54)[33], etc. They described this structure by their own terms, but the entity and the morphological features seem to be entirely same with one another. The typical profile of the organelles is parallel arrangement of elongated saccular structures containing amorphous material of very low density and the wall composed of a very thin membrane and

relatively dense small granules arranging in a layer on the outer surface of the thin membrane (Fig. 1).

These granular components of endoplasmic reticulum or ergastoplasmic granules contain abundant RNA and give most of the basophilic nature to the cytoplasm (Porter, '53, '54[17,18], Palade, '53[23], Weiss, '53[30]).

According to Porter ('54)[18] they show 100 to 300 Å in size, while de Robertis ('54)[34] reported these granules of 70 to 100 Å in dimension. In the author's measurment, they have 130 to 200 A size both in control and cancer cells. These granules correspond to the dense granules in nucleoli, nuclei and cytoplasm in their density, shape, and size. Porter called these granules "basophilic component of cytoplasm" or RNA granules, while Weiss named these "ergastoplasmic granules".

The endoplasmic reticulum or ergastoplasmic sacs change their morphological profiles secondary to the physiological conditions, functional phases, and the developmental stages of the cell (Weiss, '53)[30].

In a study of intracytoplasmic sacs, Watanabe ('55)[28] observed many types of these structures in exocrine pancreatic cells and in myeloid cells. He also assumed that these various types such as swollen, vacuolar and vesicular forms may occur from the original flattened type of the sacs by swelling and constrictions presumably in response to various functional and living states of the cell.

In embryonic cells, most of the granular component of endoplasmic reticulum or ergastoplasmic granules are present in cytoplasm being detached from the membranous component of the reticulm or sacs themselves (Howatson and Ham, '55)[35].

The epithelial cells of a normal lactating mammary gland of the "German Mouse" usually have abundant endoplasmic reticulum of typical arrangement. But, mammary cancer cells of C_3H and DBA strain mice usually have scant and atypical shapes of this structure showing many protrusions and indentations at the wall of the saccular components of the reticulum, and most of the granular components are present in cytoplasm being free from the saccular components of the reticulum as seen in embryonic cells (Fig. 3-12).

These abnormal forms of the structures seen in cancer cells may be full of suggestions for their functions which presumably are the abnormal protein synthetic activities. However, it remains still in question whether the morphological features of endoplasmic reticulum of the cancer cells and of embryonic cells are entirely same or not, and further observations and considerations concerning this problem are necessary.

4) **Mitochondria.** Mitochondria of normal lactating cells are fairly uniform and show spherical or ellipsoid shape of about 0.8 micron size (Fig. 1). They have typical internal structures of cristae mitochondriales (Palade, '52)[36] and

moderately dense amorphous stroma. The distribution of the mitochondria in normal lactating cells are fairly even in cytoplasm, but in both kinds of cancer cells, they are sometimes abundant at the sites around the virus clusters or juxtanuclear "Matrix (Gaylord) like regions" (Fig. 11, 12).

Many of the swollen, large and pale mitochondrias of monstrous shapes are also recognized (Fig., 3, 6, 12). They have irregularly destroyed or disappeared "cristae" and decreased electron density of the stroma. These atypical forms of mitochondrias and of endoplasmic reticulum are recognizeable in the same cells at the same time. In such a cell, the number of endoplasmic reticulum is fewer than that of the normal lactating cells, but mitochondrias are sometimes not fewer in number than those of the control cells (Fig. 3).

5) **Golgi's complex.** In 1953 Dalton and Felix[37] reported on the electron microscopical findings of the Golgi's apparatus with excellent electron micrographs of thin sections of the epididymis and duodenum of mice.

According to their description, Golgi's apparatus is a complex of three morphological components; horseshoe-arrangement of large vacuoli, lamellar structures without no granular component on their outer surface, and moderately dense granules of 400 Å size distributing in intimate relation with the lamellae (Fig. 1).

Recently, Weiss ('56)[38] observed electron microscopically the Golgi's complex of duodenal absorbtive cells of mice fed with cream, and recognized the active figures of this structure suggesting the activity of fat absorbtion. As to the electron microscopy of Golgi's complex of cancer cells, Haguenau and Bernhard ('55)[39] described that in some cases the Golgi's complex of cancer cells is hypertrophic, and even in the ultrastructures, it differs from that of normal cells. In the present observation, the Golgi's complex of mammary cancer cells of the mice was generally hypertrophic (Fig. 3), but even in some cancer cells showing the actual virus production, the Golgi's complex seemed not to be so hyperplastic and have no prominent structural differences with that of normal lactating cells (Fig. 7). Although the profiles of the vacuolar structures, on the outer surfaces of which many intracellular form of viral particles are being arranged, are closely resembled to those of Golgi's vacuoles in electron microscopy (Fig. 7, 8) no confirmative evidences suggesting the intimate relations between the Golgi's complex and virus production in mammary cancer cells of the mice could be found.

6) **Inclusion bodies.** Intra-cytoplasmic inclusion bodies in virus infected cells have been observed electron microscopically by many such as Gaylord ('53)[40], Dohi et al, ('53, '54)[41,42], Harford et al ('54)[43], and Amano ('55)[44]. And those in malignant mammalian tumor cells were recently studied photomicroscopically by Guerin ('55)[45] on mammary adenocarcinoma cells of mice. More recently, Takaki et al, ('56)[46] observed electron microscopically such structures closely resembling

those seen in virus infected cells even in various kinds of human malignant tumor cells. They classified the "inclusion body like structures" seen in malignant tumor cells into three types of A, B and C according to their morphological features. Most of the inclusion bodies seen in C_3H and DBA mammary adenocarcinoma cells are 1 to 10 microns or more in size and usually present one but sometimes two or more in number within a same cell. They are uaually ellipsoid, highly and homogeneously dense, and have relatively smooth outlines or clear demarcations (Fig. 6). This type of inclusion body seems to correspond with that of type A classified by Takaki et al.

While in some of them, clear limitting membranes which sometimes seem to be double nature and show certain internal structures suggestive of small and dense granules, small circular structures, mitochondria-like structures with remnants of "cristae", and debri of lamellar double membranes of endoplasmic reticulum have been found (Fig. 8). Some of the latter type of inclusion bodies also seem to correspond with those of type C of Takaki et al. In the tumor cell cytoplasm, they usually appear near the nuclei and are sometimes present in the areas close to the indented nuclear membranes (Fig. 6). In undifferentiated areas of the tumor, they frequently appear in most of the cancer cells (Fig. 6), while in well differentiated areas, they are very few or none in a plane of a section.

These inclusion bodies of mammary cancer cells of mice closely resemble those seen in cells with infection of various kinds of viruses, and are not distinguishable from virus induced one by means of electron microscopy of osmium fixed materials. The appearance of both inclusion bodies and viral clusters in a same cancer cell is not infrequent (Fig. 8). In some inclusions, interesting structures which may suggest the intracytoplasmic virus clusters arranged on the vacuolar structures were found (Fig. 8), but confirmative figures of transition between inclusion bodies and virus clusters are now lacking.

7) **Viral particles.** The electron microscopical observations of the milk agent in thin sections of spontaneous mammary carcinoma of mice were done by Bang and Andervont ('53)[9], Dmochowski et al ('53)[10] and Bernhard et al ('55)[11]. Adding to these, Fawcett and Wilson ('55)[47] observed dense homogeneous particles of 140 to 170 mμ size which show no particular internal structures in thin sections of spontaneous hepatome of C_3H mice.

Both in C_3H and DBA mammory cancer tissues of mice, two forms of viral particles are classifiable as found in the observation by Bernhard et al.

(a) **Intracellular form.** In thin sections, this form of virus shows 50 to 70 mμ (average 60 mμ) in outer diameter, and has a circular doughnut-like structure consisting of a dense limiting membrane of 10 to 20 mμ in thickness and a relatively lucent central portion of 40 to 50 mμ in diameter. Some of the particles

are evenly dense and limiting membranes are not recognizable. They sometimes appear in cytoplasm not showing characteristic features but in sparse or clustered fashion (Fig. 3, 4, 12). But frequently, they arrange on the outer aspects of aggregated several large vacuoles or sacs being bordered by very thin limiting membranes of about 10 mμ thick and showing highly lucent internal lumens (Fig. 7, 8). Within such vacuoles, few viral particles of the extracellular form are frequently recognizable (Fig. 7).

(b) **Extracellular form.** In intercellular spaces and in ductal lumens of the carcinoma, many of the extracellular form of viral particles are seen (Fig. 3, 5, 9). This form of virus is spherical or ellipsoid in shape and mostly 100 mμ in size. They are moderately dense or relatively lucent particles with a dense limiting membrane of about 10 mμ thick and a dense central body or " Nucleoid " (Morgan, '54)[48] of 40 to 50 mμ in diameter. The nucleoid is spherical and usually situated in the central region of a relatively lucent viroplasm but, in some, it situates rather eccentrically on the inner aspect of the limiting membrane. These shapes of viral particles well correspond to those of many pox-group viruses of the proliferating stage (Gaylord, '53[40], Morgan, '54[48], Suzuki et al, '56[49]).

(c) **Mode of escape of viral particles into ductal lumens.** On the cell membranes of the two kinds of cancer cells facing the ductal lumens or intercellular spaces, there are many microvilli. And within the distal part of these microvilli, the intracellular form of viral particles are frequently seen (Fig. 5). This figure was also observed by Bang and Andervont ('53)[9] and Bernhard et al ('55)[11], and these may suggest the mode of passage of viral particles through the cell membrane into the extracellular spaces without destroying the host cell.

These two forms of viral particles could be found here and there in many sections of both strains of cancer cells, but they could not be found in those from the normal loctating cells of the German Mouse (Fig. 1, 2), and there are no structural, dimensional and distributional differences between these viruses of the corresponding two forms seen in the C_3H and DBA strain mammary cancer tissue.

8) **Intracellular ductulus-like spaces.** In some of the tumor cells there is a space bordered by a thin smooth limiting membrane, which seems to be identical with the cell membrane in thickness and in density, and shows sometimes a small number of microvilli-like protrusions on the inner surface. Usally, in such spaces, many of the viral particles of extracellular form are recognizable, but no other particular structures excluding the usual cytoplasmic elements were seen on the outer aspect of the limiting membrane of these spaces (Fig. 3). Such spaces have also been recognized in the electron microscopic study of sectioned Ascites Hepatoma Cells of Yoshida grown in omentum of a rat (Takaki et al, '56)[50], and these spaces in mouse mammary cancer cells and in Ascites Hepatome Cells of rat

seem to be identical.

However, the relations between these spaces and the ductal lumens or Golgi's vacuoles in both cancer cells are now obscure and these should be confirmed by serial sections or many electron micrographs in further studies.

9) **Central fine granular regions (CFGR)** (Suzuki). Some of the tumor cells show a large central area consisting of diffusely fine and granular material and has no clear demarcation at the border. Few mitochondrias and endoplasmic reticulum are seen around the region (Fig. 10).

These structures, seemingly, resemble those structures seen in the virus infected cells and named as "Matrix" (Gaylaord, '53)[40], "Aggregation of fine granular material" (Morgan, '54)[48] and "Virus induced necrotic region (VINR)" (Friedlaender et al., '55)[51], During the electron microscopy, the cells which have these regions could be observed about one per 2 to 4 holes of 200 mesh-grid. Takaki et al. ('56)[46] also observed "VINR-like regions" and "Matrix-like regions" together with "Inclusion body-like structures" and "Virus-like particles" within human malignant tumor cells. But these structures suggesting viral infection are always accompanied by the real presence of the cell nuclei and virus or "Virus-like particles" within the same cells. In CFGR of C_3H and DBA mice mammary cancer cells, no accompanying nuclei and viral particles have been seen in the same cells of many sections, besides, the morphological features of the fine granular material of CFGR are rather akin to those of the nucleoplasm. Hence, it is very reasonable to consider that the CFGR is an electron microscopic figure of a stage in mitosis, but such a kind of figure was not recognized in Selby's observation ('53)[52] on the mitosis of Ehrlich mouse ascites tumor cells, and the author has also not encountered such figure of mitosis in the electron microscopy of many tumor cells.

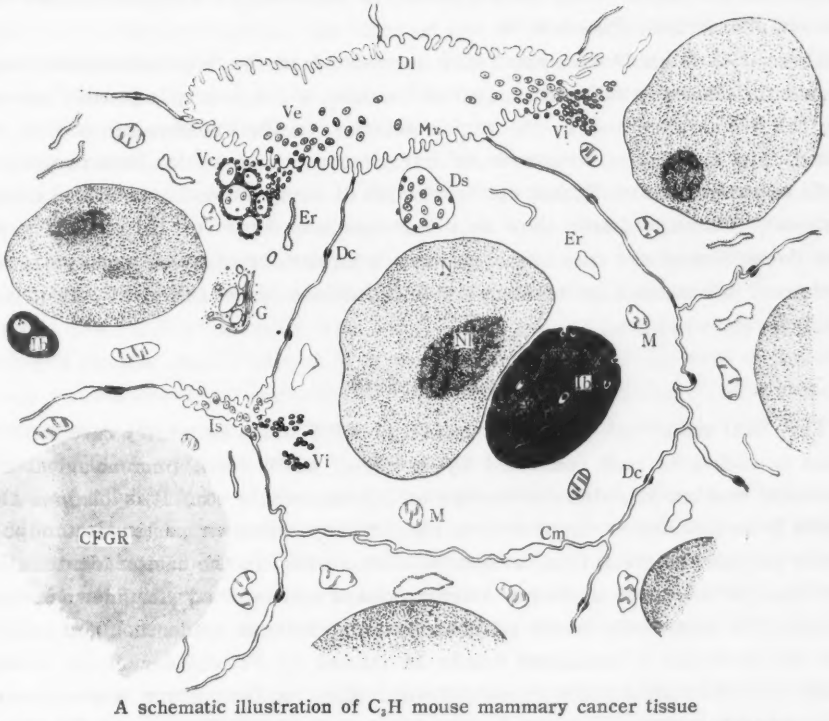
The CFGR also seems to be different with VINR in the feature of the consisting fine granules, presence of no nucleus within a same cell, and the vague demarcation from the surrounding cytoplasm.

The "Aggregation of fine granular material" or "Matrix" in virus infected cells has been understood as an area where the viral particles actually occur and proliferate, but the CFGR being discussed, shows different features of consisting fine granules, and up to date, the author has not seen any newly formed virus-like structures in or around the CFGR of many electron micrographs taken from many sections of these tumors.

10) **Matrix-like regions.** The juxtanuclear regions of some mammary cancer cells of the mice, at which the nuclear membranes usually show shallow indentations into the nucleoplasm, aggregation of relatively dense fine granular material showing irregular forms and vague demarcations and accompanying the surround-

ed aggregations of many mitochondrias could be observed (Fig. 11, 12). These figures were found about one per several holes of 200 mesh-grid during the observation of many sections.

Such figures have also been observed by Gaylord ('53)[40], Morgan ('54)[48], and Suzuki et al ('56)[49] in virus infected cells and named "matrix". Also, they have been found by Takaki et al ('56)[46,50] and Hashimoto et al ('56)[53] in human and mammalian cancer cells. But this structure is different electron microscopically from VINR (Friedlaender) or "CFGR" already mentioned. During the present observations, the author found only one figure in which the viral particles of intracellular form appear in a "Matrix-like region" (Fig. 12), and this may



A schematic illustration of C_3H mouse mammary cancer tissue

NI: Nucleolus N: Nucleus Er: Endoplasmic reticulum M: Mitochondria G: Golgi's complex Ib: Inclusion body Is: Intercellular space filled with viral particles of extracellular form. Vi: Viral particles of intracellular form. Ve: Viral particles of extracellular form. Vc: Viral cluster Mv: Microvilli; some of them contain intracellular form of viral particles within the distal portion of them. Dl: Ductal lumen Ds: Intracellular ductulus-like space Dc: Partial increase of density in cell membrane and in adjuscent cytoplasmic stroma. Cm: Cell membrane CFGR: "Central fine granular region"

suggest the occurrence of viral particles from this region. The other "Matrix like regions" in which no viral particles could be found may represent figures in the latent stage of virus reproduction, or the absence of viral particles might be due to the different height of the cutting plane, then the final conclusion should be made by the observations of many serial electron micrographs.

11) **Cell membranes.** At the borders of both kind of tumor cells, where they are in contact with one another, parallel arrangement of two cell membranes about 10 to 13 mμ thick are recognizable (Fig. 6, 7). Between these two cell membranes, there are intercellular spaces of about 20 to 30 mμ in width, and in some sites where many viral particles of extracellular form are often present, the spaces are wide, irregular in form and usually show few microvilli on the outer surfaces of the cell membranes (Fig. 4, 6, 9).

These wide intercellular spaces may correspond to the intercellular secretory canaliculi. While in other sites, partial increase of the density in parallel arranging cell membranes and in the region adjacent to the cytoplasmic stroma are noted (Fig. 6). These structures of cell membranes have also been recognized both in normal and malignant epithelial cells of many kinds (unpublished observations). They essentially show no differences with those of the normal cells, but the outline of cell membranes is more irregular than that of the normal cells, and many indentations, protrusions and invaginations of the cell membranes could often be observed (Fig. 6).

Discussion

The viral nature of Milk agent in C_3H or DBA strain mammary carcinoma of mice is said to be well confirmed by isolation, purification, immunological and chemical studies, experimental studies on it's occurrence etc. It is obvious that there is no absolute or direct way to identify any unknown particulate to be a virus particles of given type in electron micrograph. In the case of identification of virus particles seen in electron micrographs of cells infected with known isolated virus, it is necessarily based on circumstantial indirect evidence. The criteria for the evidences is postulated briefly as follows by Friedländer et al. a) that viral particles should show consistent size, shape and structure when present, characterizing the particular viruses, b) that particles should appear in appropriate numbers in infected cells but should not be present in appropriate controls, c) that under conditions of fixations with osmic acid as described, the particles should show on excessive electron density compared with other cytoplasmic or nuclear particles of similar size. In the case of milk agent in the studies the author would like to add furthermore that the size of the particles found in electron micrographs should have the same size as that of isolated and purified

milk agent taken from the same strain of high mammary carcinoma incidence.

On the study of the size of abnormal particles, Graff et al. ('47)[6] reported on the "heavy particles" of about 100 mμ size seen electron microscopically in the centrifugate of the high cancer strain milk. Bernhard et al. ('55)[11] also reported on intra- and extra-cellutar suspicious particles with diameters of 65 mμ and 100 mμ, respectively, showing the same morphology with those described in this study in thin sections of mice mammary cancer. Though the result of Passey et al. ('47)[7] who reported the particles of 20 mμ size in the water extract of the cancer cells of high cancer strain mice, might be too small to assume them as milk factor virus itself, the sizes of the viral particles measured in the present study are well corresponded with those of the above authors' results. However, as described in the introduction, the size and the structure of the extracellular viral particles reported by Porter and Thomson ('48)[8] are somewhat larger than those of the above authors and the present study, and the difference in size may be due to the differnce in technical method.

The extracellular form of the viral particles, which are assumed to be Bittner's milk agent itself, show the typical figure of "nucleoid" structure as described by Morgan et al. ('54)[48]. Excluding their size, they closely resemble the proliferating viral particles of some of the pox group viruses such as vaccinia, fowl pox, molluscum contagiosm and etc. But the mode of the occurrence in cancer cells is not entirely the same with those of pox group viruses, and this type of Bittner's virus is usually seen only in extracellular spaces, while most of the pox group viruses showing "nucleoid" are seen in host cell cytoplasm. In the present observations, the structural changes of nucleoid as seen in some pox group viruses (Morgan, '54)[48] were not found in Milk agent in situ.

In intracellular form of the agent, homogenous electron dense particles and doughnut shaped one with pale central core and dense outer shell were seen which are also common in various kinds of other viruses. The author has observed many of the viral particles in undifferentiated areas of the mammary cancer tissue, but generally, they were few in number in relatively well differentiated areas.

On the proliferation of pox group viruses, an "aggregation of fine granular material" (Morgan, '54)[48] or "Matrix area" (Gaylard, '53)[40] can be observed within the host cell cytoplasm prior to the occurrence of the viral particles, and the prototype of viral particles is recognized in or around these areas. In mammary cancer cells of the mice, the author has observed only one figure in which the viral particles were occurring actually in the "Matrix like region". This figure will give enough proof to the assumption that the viral particles can occur from these "Matrix like regions", but such figure showing the viral pro-

liferation within it is now so few that the author would like to call it a "Matrix like region" rather than a "Matrix region". In "CFGR" however, no suspicious abnormal particles associated with this region could be observed even in many sections of the both strain mammary cancer cells. There are no articles referring to this type of change and the relationship between "CFGR" and viral particles now remains in question. The author has not attained yet the solution on the problem of the nature and the significance of "CFGR" and on the relations between this structure and VINR (Friedlaender, '55)[51] at present. These problems and questions will be settled by further observations with histo- and cytochemical considerations.

On the observations of inclusion bodies as well as in "CFGR", we may also have the same kind of problem in their identification for viral origin as in the case of viral particles. The same criteria might be able to be used here, too. Photomicroscopically, there exist many spherical or ovoid bodies of 1 to 10 microns size which show smooth outlines within the cancer cells. These bodies are histochemically, Feulgen's reaction positive, methylgreen staining positive and Oil red O staining negative, and situated in the region close to the nucleic membranes of the cancer cells. Such kind of bodies were already observed by Guérin ('55)[45] photomicroscopically in mammary cancer cells of mice and were assumed to be inclusion bodies. It will be natural to consider that the electron dense large bodies seen within cancer cells are really inclusion bodies because of their size and shape which are indistinguishable from those seen in non-tumer genic virus infections of cells. The results of histochemical reactions for nucleoprotein of desoxyribose type are also compatible with this view.

In the electron microscopic observations of sectioned mammary cancer cells of mice, Bernhard et al. ('55)[11] demonstrated an electron micrograph of a juxta-nuclear viral cluster as an inclusion body. Although it is influential to consider that viral particles originates from the inclusion body, and as a matter of fact, close relationships have been known in the occurrence of viral particles of many pox group viruses such as vaccinia, ectromelia, molluscum contagiosum and etc., such a type of viral cluster was distinguished from the inclusion body described in the present study, because the author has frequently observed large, highly dense and homogeneous bodies with defined margins at the juxta-nuclear region of many cancer cells.

In this paper, these homogeneously dense bodies were described as inclusion body. This type of inclusion body seems to correspond to those of Type-A observed by Takaki et al. ('56)[46,50] in the electron microscopy of human malignant tumor cells.

Then, what is the nature and the significance of the inclusion bodies observed

within mammary cancer cells of the mice? In some electron micrographs of inclusion bodies, figures suggestive of viral clusters arranging on the outer surfaces of vacuolar structures were demonstrated, and these figures may suggest the intimate relation between viral clusters and inclusion bodies in their origination (Fig. 8).

The viral particles and the other cellular structures described in the present study were found in the spontaneous mammary cancer cells of C_3H and DBA strain mice, and no qualitatively essential differences could be found in electron microscopy of the sections of the both strain mammary cancer. However, notwithstanding their same grade of differentiation, viral particles were, generally, seemed to be numerous in C_3H cancer tissue rather than that of the DBA strain mice.

All of the changes described and discussed above such as virus particles, Inclusion bodies and Matrix like regions are indistinguishable from those seen in non-tumorgenic virus infected cells electron microscopically. They also fulfill the criteria for identification of viral origin. Other changes such as those in nucleus, endoplasmic reticulum and mitochondria are also compatible with changes seen in human cancer cells. The present study seems to confirm the viral nature of C_3H and DBA breast carcinoma morphologically. It also suggests much in evaluation of findings in electron microscopy of human cancer cells (Takaki)[46,50].

Although we have not clarified the real role of the milk agent in occurrence of breast carcinoma in C_3H and DBA strain mice, further studies on electron microscopy, serial and combined to histochemical procedure, will give further knowledge on this problem.

Summary

1. An electron microscopic observation on the ultrastructures of spontaneous C_3H and DBA strain mice mammary cancer were carried out by the use of ultrathin sectioning method.

2. No morphologically essential differences of the viral particles and the cell structures between the both strains of mammary cancer could be recognized.

3. Intracellular and extracellular forms of virus could be observed within both strains of cancer cell cytoplasm, ductal lumens and intercellular spaces.

In thin sections, the intracellular form of virus is highly dense and shows mostly doughnut-like structure of 60 mμ size, while the extracellular form is ovoid or spherical in shape, 100 mμ in size and showing a nucleoid of 50 mμ size in its center.

4. The extracellular form of the virus seems to correspond with Bittner's milk agent.

5. Other cellular structures which are similar to the changes as seen in virus infected cells such as inclusion bodies, "Matrix like regions" and "Central Fine Granular Regions" were also observed and discussed.

6. Further detailed electron-microscopic observations on spontaneous and experimentally induced mice mammary cancer should be important in the service for ultramorphological comprehension of "Human Cancer".

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References

1. Staff of the Roscoe B. Jackson Memorial Laboratory per C.C. Little; Existence of none-chromosomal influence in incidence of mamary tumors in mice. Science, 78: 465, 1933.

2. Bittner, J.J.; Some possible effects of nursing on mammary gland tumor incidence in mice. Science, 84: 162, 1936.

3. do.; The milk-influence of breast tumors in mice. Science, 95: 462, 1942.

4. Bryan, W.R., Kahler, H., Shimkin, M.B., and Andervont, H.B.; Extraction and ultra-centrifugation of mammary tumor inciter of mice. J. Nat. Cancer Inst., 2: 451, 1942.

5. Visscher, M.B., Green, R.G. and Bittner, J.J.; Characterization of milk influence in spontaneous mammary carcinoma. Proc. Soc. Exp. Biol. and Med., 49: 94, 1942.

6. Graff, S., Moore, D.H., Stanley, W.M., Randall, H.T., and Hoagensen, C.D.; Program of the 4th International Cancer Research Congress, St. Louis, 144, 1947.

7. Passey, R.D., Dmocnowski, L., Astbury, W.T., and Read, R.; Electron microscope studies of high- and low-breastcancer strains of mice. Nature, 160: 565, 1947.

8. Porter, K.R. and Thompson, H.P.; A particulate body associated with epithelial cells cultured from mammary carcinomas of mice of a milk factor strain. J. Exp. Med. 88: 15, 1948.

9. Bang, F.B., and Andervont, H.B.; Detection of the mammary tumor inciter (MTI) in thin sections of spontaneous mouse tumors. J. Appl. Physics. 24: 1418, 1953.

10. Dmochowski, L., Cushman, D., Haagenzen, and Moor, D.H.; A study in the electron microscope of thin sections of normal and malignant mammary tissues of mice. J. Appl. Physics, 24: 1418, 1953.

11. Bernhard, W., Bauer, A., Guérin, M. et Oberling, C.; Etude au microscope électronique de corpuscules d'aspect virusal dans des épitheliomas mammaires de la souris. Bull. Ass. fr. Cancer. 42: 163, 1955.

12. Latta, H. and Hartmann, J.F.; Use of glass edge in sectioning for electron microscopy. Proc. Soc. Exp. Biol. Med. 74; 436, 1950.

13. Porter, K.R. and Blum, J.; A study in microtomy for electron microscopy. Anat. Rec. 117: 685, 1953.

14. Berhard, W., Bauer, A., Gropp, A., Haguenau, G., and Oberling, C.; L'ultrastructure du nucléole de cellules normales et cancéruses; étude au microscope électronique. Exp. Cell Res. 9: 88, 1955.

15. Selby, C. C.: Electron micrographs of mitotic cells of tne Ehrlich mouse ascites tumor in thin sections. Exp. Cell Res. 5: 386, 1953.

16. Porter, K. R., Claude, A. and Fullam, E. F.; A study of tissue culture cells by electron microscopy. J. Exp. Med., 81: 233, 1945.

17. Porter, K. R.; Observation on a submicroscopic basophilic component of cytoplasm. J. Exp. Med. 97: 727, 1953.

18. do.; Electron microscopy of basophilic components of cytoplasm. J. Histochem. and Cytochem. 21: 346, 1954.

19. Dalton, A. J., Kahler, H., Striebich, M. J. Lloyd, B. J.; Finer structure of hepatic, intestinal and renal cells of the mouse as revealed by electron microscope. J. Nat. Cancer Inst., 11: 439, 1950.

20. Dalton, A. J.; Electron micrography of epithelial cells of the gastro-intestinal tracts and pancreas. Am. J. Anat. 89: 109, 1951.

21. Dalton, A. J. and Striebich, M. J.; Electron microscopic studies of cytoplasmic component of some of the cells of the liver, pancreas, stomach, and kidney following treatment with ribonuclease. J. Nat. Cancer Inst. 12: 224, 1951.

22. Palade, G. E.; A study of fixation for electron microscopy. J. Exp. Med. 95: 285, 1952.

23. do.; Asmall particulate component of the cytoplasm. J. Appl. Phys. 24: 1419, 1953.

24. do.; Studies on the endoplasmic reticulum. II. Simple dispositions in situ. J. Cytology. (in press).

25. Palade. G. E. and Porter, K. R.; The endoplasmic reticulum of cells in situ. Anat. Rec. 112: 68, 1952.

26. do; Studies on the endoplasmic reticulm. I. Its identification in cells in situ. J. Exp. Med. 100: 641, 1954.

27. Watanabe, Y.; On the structure of cytoplasm. 1st Symposium of Japanese Soc. of Electron microscope. Nov., 1953, Osaka.

28. do.; A study on the intracytoplasmic sacs in exocrine pancreatic cells and in myeloid cells. J. Electron microscopy. 3: 43, 1955.

29. Gautier, A. and Diomede-Fresa, V.; Etude au microscope électronique de l'ergastoplasm des glandes salivaires du rat. Mikroskopie, 8: 23, 1953.

30. Weiss, J. M.; The ergastoplasm; Its fine structure and relation to protein synthesis as studies with the electron microscope in the pancreas of the swiss albino mouse. J. Exp. Med. 98: 607, 1953.

31. Rinehart, J. F. and Farquhar, M. G.; Electron microscopic studies of the anterior pituitary gland. J. Histochem. and Cytochəm. 1: 93, 1953.

32. Sjöstrand, F. S. and Rhodin, J.; The ultrastructure of the proximal convoluted tubulus of the mouse kidney as reveal by high resolution electron microscopy. Exp. Cell Res. 4: 426, 1953.

33. Challice, C. E. and Lacy, E.; Fine structure of exocrine cells of the pancreas. Nature, 174: 4442, 1954.

34. de Robertis, E.; The nucleocytoplasmic relationship and the basophilic substance (Ergastoplasm) of nerve cells (Electron microscope observations). J. Histochem. Cytochem. 2: 341, 1954.

35. Howastron, A. F. and Ham, A. W.; Electron microscope study of sections of two rat liver tumor. Cancer Res. 15: 62, 1955.

36. Palade, G. E.; The fine structure of mitochondria. Anat. Rec. 114: 247, 1952.

37. Dalton, A, J. and Felix, M. D.; Studies on the Golgi substance of the epithelial cells of the epididymis and duodenum of the mouse. Am. J. Anat. 92: 277, 1953.

38. Weiss, J. M.; The role of the Golgi complex in fat absorption as studied with the electron microscope with observations on the cytology of duodenal absorptive cells. J. Exp. Med. 102: 775, 1955.

39. Haguenau, F. and Berhard, W.; L'appareil de Golgi dans les cellules normales et cancereuses de vertebres; rappel historique et étude au microscope électronique. Arch. anat. micro. Par. 44: 27, 1955.

40. Gaylord, W. H. and Melnick, J. L.; Intracellular forms of pox-viruses as shown by the electron microscope (Vaccinia, Ectromelia, Molluscum contagiosum). J. Exp. Med. 98: 157, 1953.

41. Dohi, S., Kishi, T. and Amano, S.; Studies on the fundamental forms of viral infection. VII. Pathology of virus infected cell. Transac. Soc. Path. Jap. 42 G: 118, 1953.

42. Dohi' S., Iwai, H., Hashimoto, S. and Amano, S.; Studies on the fundamental forms of viral inflammation. Report VIII. On the viral multiplication within the cells. Transac. Soc. Path. Jap. G. 43: 499, 1954.

43. Harford, C. G., Hamlin, A. and Parker, E.; Electron microscopy of early cytoplasmic changes due to influenza virus. J. Exp. Med. 101: 577, 1955.

44. Amano, S.; Pathology of virus infection. Nippon Rinshyo, 13: 1569, 1955.

45. Guérin, M.; Corps d'inclusion dans les adénocarcinomes mammaires de la souris. Bull. Ass. fr. Cancer. 42: 14, 1955.

46. Takaki, F., Suzuki, T., Yasuda, H., and etc.; Electronmicroscopic studies on malignant tumor cells. (Gann; The Japanese Journal of Cancer Research, in press).

47. Fawcett, D. W. and Wilson, J. W.; A note on the occurrence of virus like particles in the spontaneous hepatoma of C 3 H mice. J. Nat. Cancer Inst. 15: 1505, 1955.

48. Morgan, C., Ellison, A. E., Rose, H. M. and Moore, D. M.; Structure and development of viruses as observed in the electron microscope. II. Vaccinia and fowl pox viruses. J. Exp. Med. 100: 301, 1954.

49. Suzuki, T., Kan, R., Taguchi, S., Sadatsuki, H., Sasao, M., Takaki, F. and Yasuda, H.; Electron microscopic study on molluscum contagiosum. (Electron-microscopy, in press)

50. Takaki, F., Yamaguchi, H., Muramatsu, H., Hosokawa, K., Suzuki, T. and Yasuda, H.; Electronmicroscopic studies on malignant tumor cells. (Electron-microscopy, in press)

51. Friedlaender, M., Moore, D. H., Love, R., Brown, R. A. and Koprowski, H.; Studies with the electron microscope of virus-host relationships in Ehrlich ascites tumor cells I. The identification and structure of Anopheles A virus' J. Exp. Med. 102: 361, 1955.

52. Selby, C. C.; Electron micrograph of mitotic cells of the Ehrlich mouse ascites tumor in thin sections. Exp. Cell Res. 5: 386, 1953.

53. Hashimoto, M., Fuse, U. and Muroya, K.; Electron microscopic studies on the hepatoma induced by D. A. B. (Dimethylaminoazobenzene) in rat. Electron-microscopy, 4 (3): 21, 1956.

54. Bittner, J. J. and Kirschbaum, A.; Assay of methylcholanthrene-induced mammary tumors of mice for the mammary tumor milk agent, Proc. Soc. Exp. Biol. Med. 74: 191, 1950.

Explanation of Electron micrographs

Fig. 1. An epithelial cell of normal lactating mammary gland of the German Mouse. A nucleus (N) with hypertrophied nucleolus (Nl), oval mitochondrias (M) with cristae and rather dense homogeneous stroma, a Golgi's complex (G), many of the flattened-saccular type of endoplasmic reticulum (Porter) or intracytoplasmic sacs (Watanabe) (Er) appearing in a pa-

rallel arrangement, and a part of ductal lumen (Dl) are seen. 19,800 x

Fig. 2. Normal lactating mammary gland of the German Mouse. A lactating cell are being compressed, and the wide ductal lumen (Dl) is filled with many, dense, spherical fat droplets various sizes together with relatively electron-lucent amorphous substance. S: Stroma Mv: Microvilli on the surface of epithelial cell. 9,600 x

Fig. 3. Low power view of C_3H mammary cancer. Five cancer cells are facing the ductal lumen (Dl) which are filled with many viral particles of extracellular form with nucleoids (Ve) and fine granular amorphous substance. Within a surrounding cancer cell cytoplasm (at the right of the figure), there are hypertrophied Golgi's complex (G), irregular shape of mitochondrias (M), atypical shape of decreased endoplasmic reticulum (Er) and clusters of viral particles of intracellular form (Vi) are recognized. Another cancer cell (left-lower part of the figure), shows an intracellular ductulus-like space (Ds) filled with many viral particles of extracellular form. 22,000 x

Fig. 4. A DBA strain mammary cancer forming a wide intercellular space between two cells. Viral particles of intracellular form (Vi) and of extracellular form (Ve) are found in cytoplasm and the space (Is). The structural featueres of the both forms of viral particles seem to be identical with those seen in mammary cancer of C_3H strain mice. N: Nucleus 18,000 x

Fig. 5. High power view of the cell surface of C_3H mammary cancer facing the ductal lumen (Dl). Dough-nut shaped viral particles within the distal portion of some microvilli (Mv) can be noted. These figures may suggest the mode of escape of the viral particles from the host cell into the ductal lumen. Ve: Viral particks of extracellular from. M: mitochondria. Er: Endoplasmic reticulum. 27,200 x

Fig. 6. Low power view of mammary cancer cells of a C_3H strain mouse; Four inclusion bodies (Ib) are seen at the juxta-nuclear regions of the four neighbouring cells. These cancer cells have generally scant and atypical shaped endoplasmic reticulum and mitochondrias. They also show irregular course of cell membranes with partial increase of density in parallel arranged cell membranes (Dc). The network-like or the tangled string-like aggregates of nucleolar substance (Nl) are noted but this figure is presumably not characteristic. 9,100 x

Fig. 7. A mammary cancer cell of C_3H strain mouse; a juxta-nuclear viral cluster associated with vacuolar structures is illustrated. Many viral particles of Intracellular form are seen around and on the outer surfaces of these vacuolar structures. Golgi's complex (G) is also recognizable at the right upper corner. 24,500 x

Fig. 8. Three mammary cancer cells of a C_3H strain mouse are shown in this figure. The upper cell has a viral cluster (Vc_1) consists of many viral particles of intracellular form arranged on the outer aspects of many vacuolar structures and a dense inclusion body (Ib) which may suggests genetically an intimate relation with viral cluster. Note the two vacuolar structures in the inclusion body, these have small and dense ring-form bodies around them and are closely resembling with those of the adjacent viral cluster. The lower cell also shows another viral cluster (Vc_2) in the juxta-nuclear region. Generally, these cancer cells have few, pale and irregular shape of mitochondrias (M) and scant endoplasmic reticulum (Er). 30,000 x

Fig. 9. High power view of C_3H mammary cancer; an intercellular space filled with many viral particles of extracellular form (Ve). Note the dense and spherical central bodies or "Nucleoids" in many viral particles. 28,000 x

Fig. 10. A "Central fine granular region" (CFGR) in a mammary cancer cell of a C_3H strain mouse. The central cell shows no nucleus and seems to be expanded. The cell center

is mostly occupied by diffuse proliferation of fine granular amorphous material. Few mitochondrias (M) and scant endoplasmic reticulum (Er) are seen around it, but no viral particles can be found. 11,800 x

Fig. 11. A juxta-nuclear "Matrix-like region" in a mammary cancer cell of a C_3H strain mouse. In this figure, the region consists of fine, dense and homogeneously granular material and the surrounding aggregates of many mitochondrias. Although some vacuolar structures and small dough-nut shaped particular structures are seen in the fine granular material, no distinct viral particles can be seen in this region. 22,800 x

Fig. 12. Another "Matrix-like region" showing many dough-nut shaped viral particles and the surrounding fine granular dense material. 35,200 x

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電子顕微鏡的細胞組織病理学 (III)
マウス自然発生乳癌の電子顕微鏡的研究

鈴木昭男 (慈恵医大病理学教室)

近年に至り C_3H マウス乳癌発生に際し重要な意義を有する乳因子が Virus 性の性格を示すことが種々の点から確認されてきている。著者は C_3H 及び DBA 両系マウス乳癌細胞を超薄切して電子顕微鏡下に観察し，両系乳癌細胞中に Virus と考えられる細胞内型及び細胞外型の二種の異常粒子を認めた他，種々の非腫瘍形成 Virus の感染を示す細胞にしばしば認められる如き各種の変化乃至構造と酷似する電顕像を多数認めこれらについても記載及び考察を加えた。

本研究の結果からすれば，明らかに悪性腫瘍と考えられる C_3H 及び DBA 両系マウス自然発生乳癌において認められる種々な変化乃至構造は他の各種の非腫瘍形成性 Virus 感染細胞に認められるそれらと全く電子顕微鏡形態学的には区別し得ず，悪性腫瘍発生と Virus 感染との間に横たわる種々の問題を闡明する上にはなはだ興味ある事実と考えられる。


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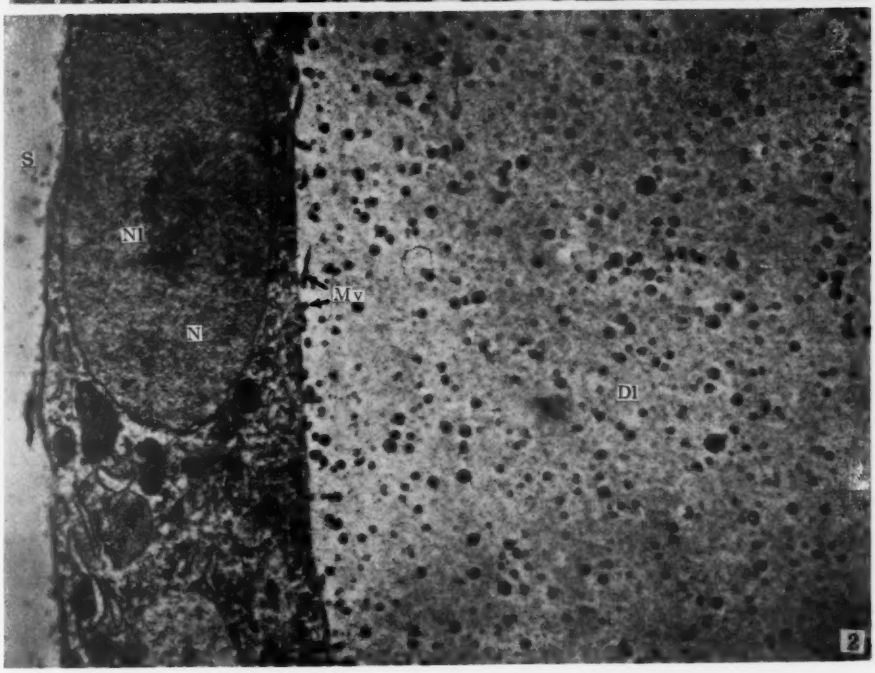
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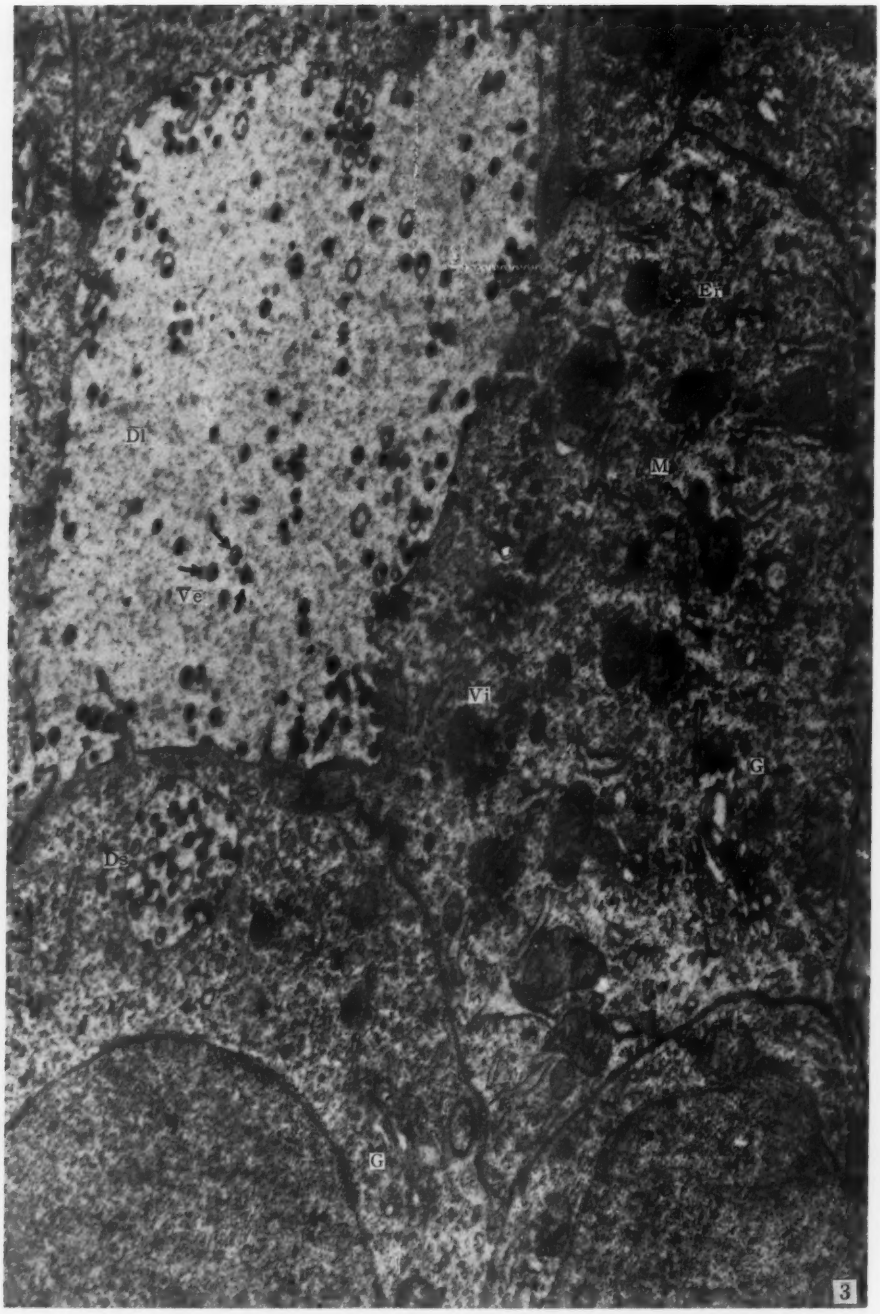
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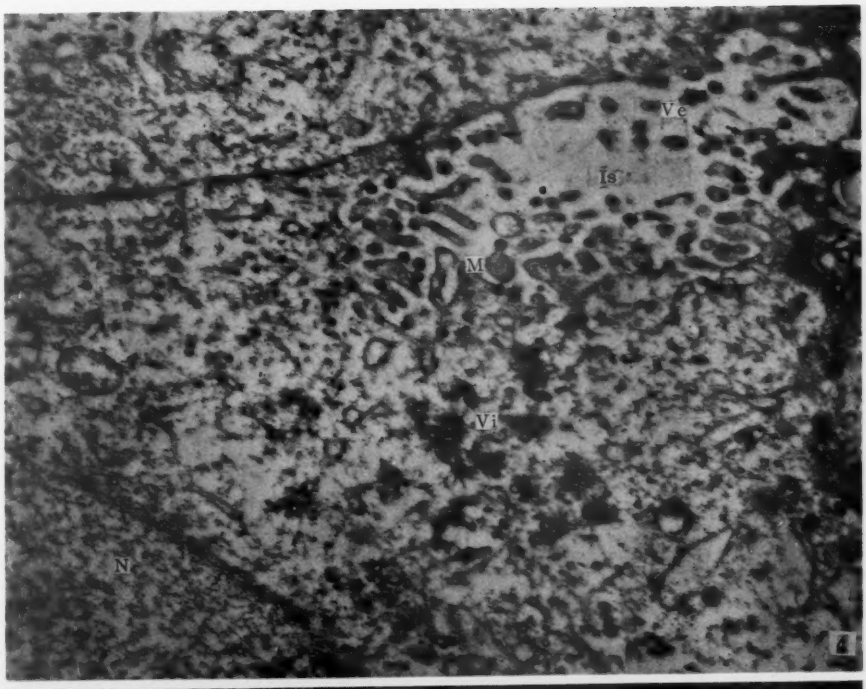
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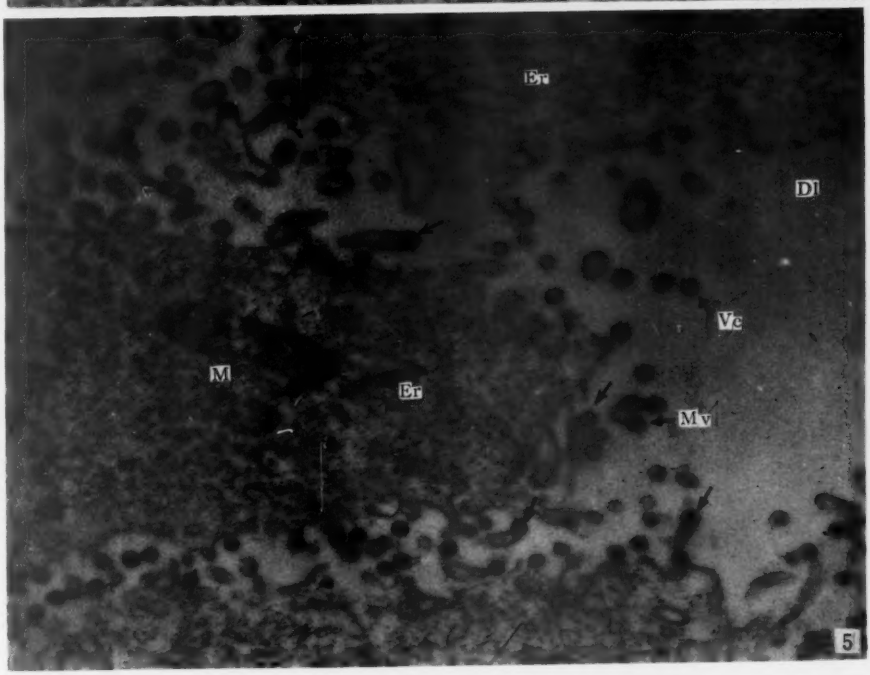
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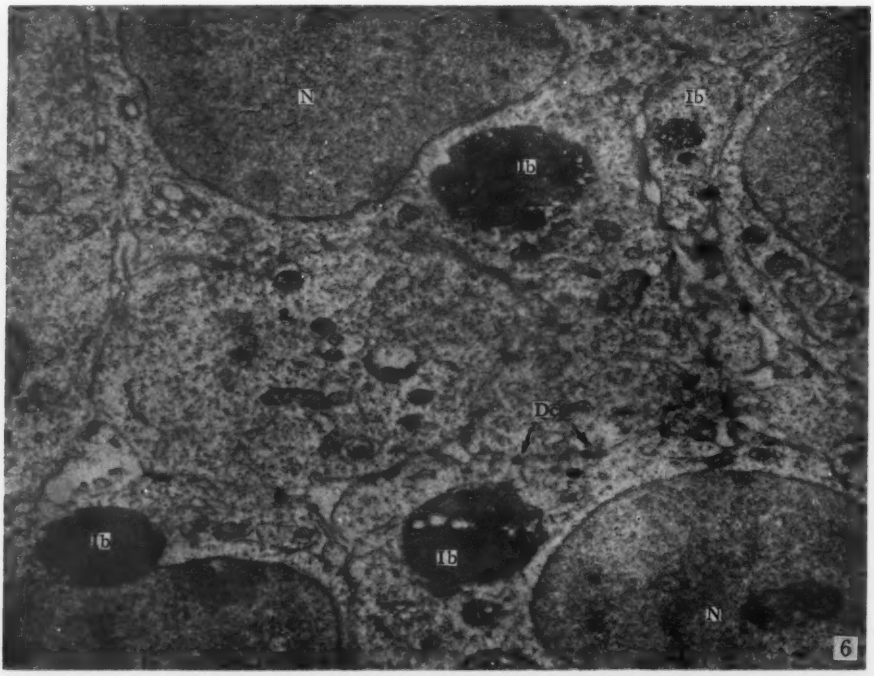
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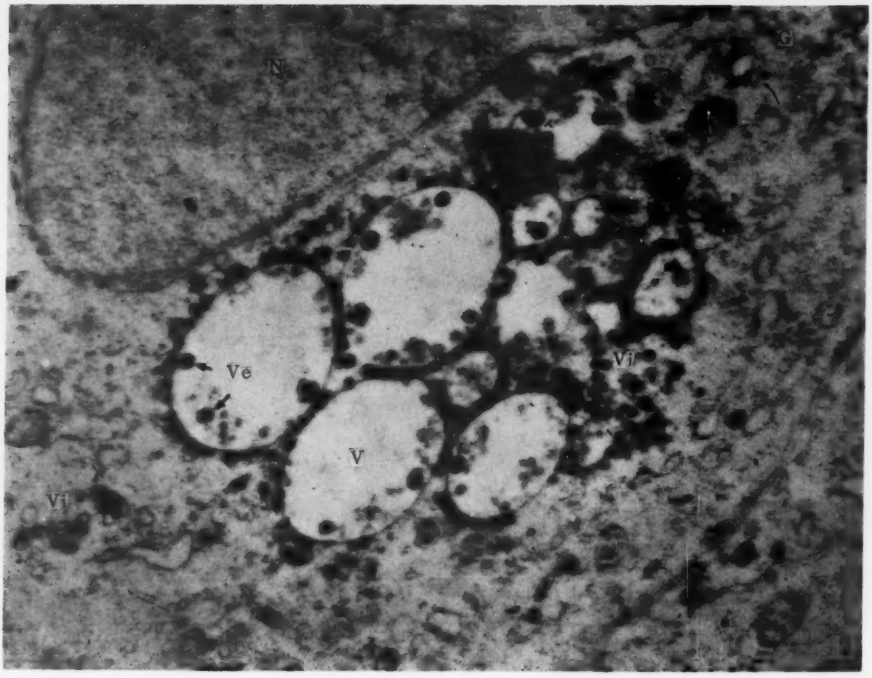
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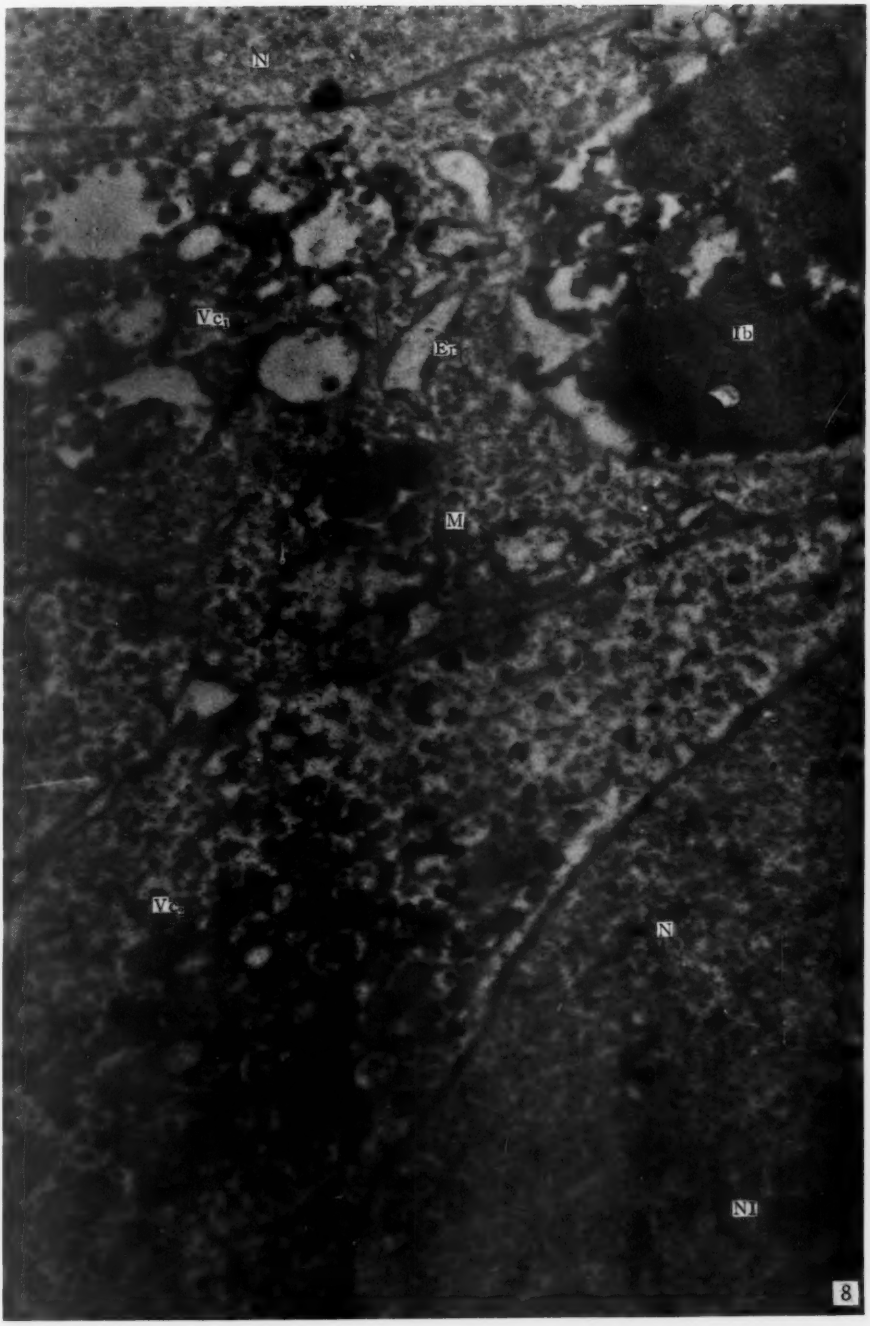
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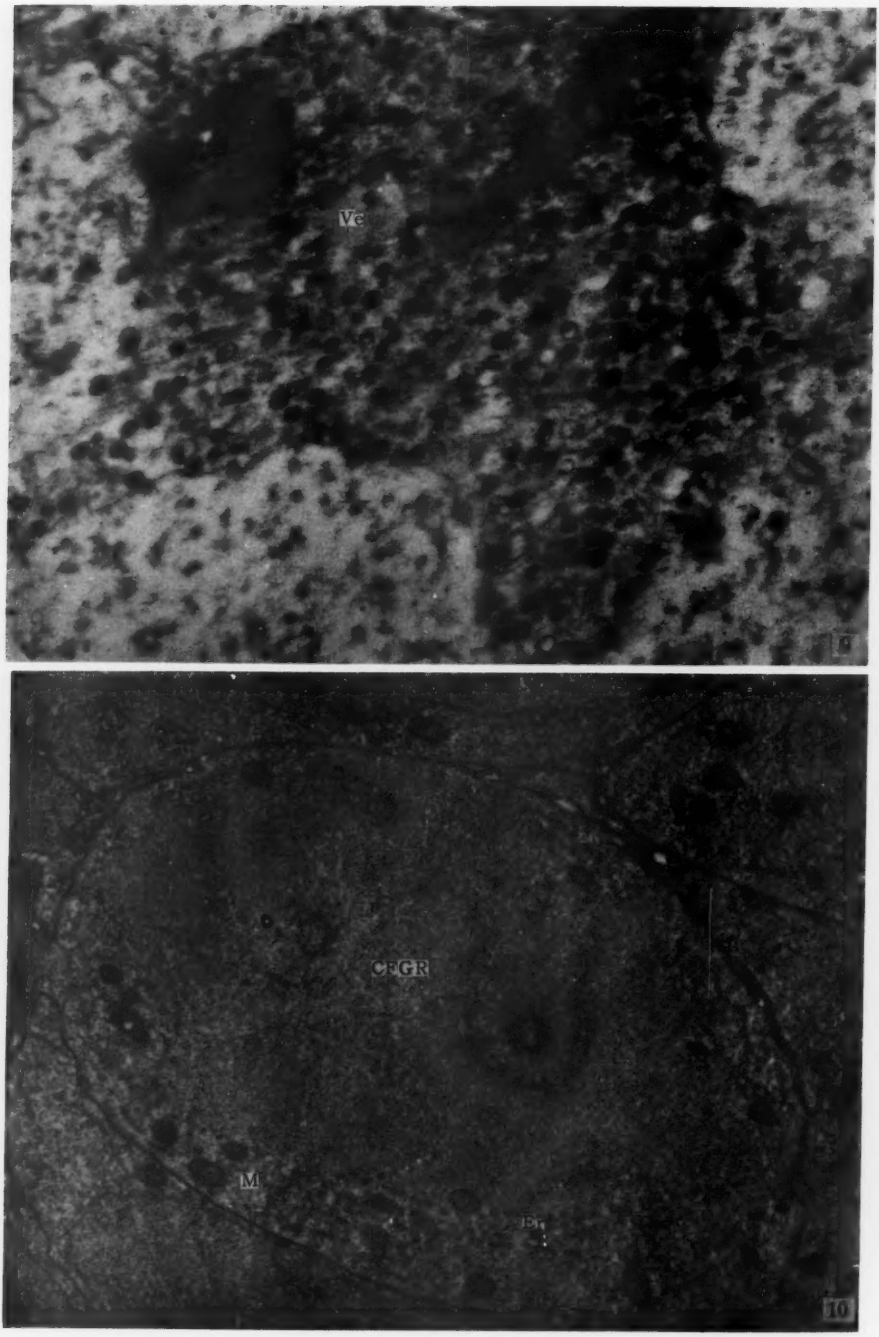
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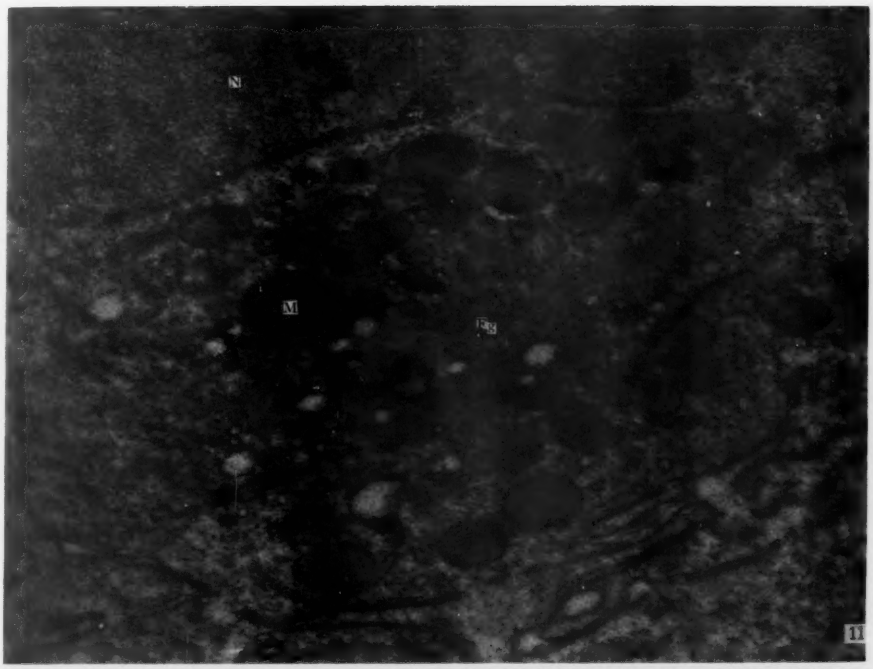
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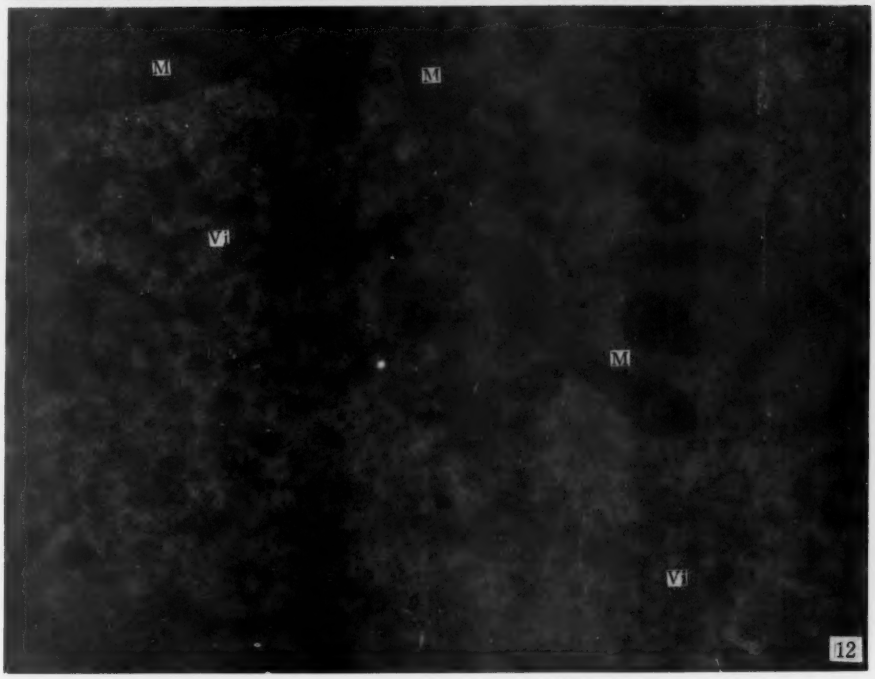
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[GANN, 48, 57~64; March, 1957]

SCREENING TESTS OF VARIOUS CHEMICAL SUBSTANCES AS CARCINOGENIC HAZARDS (Report 1)

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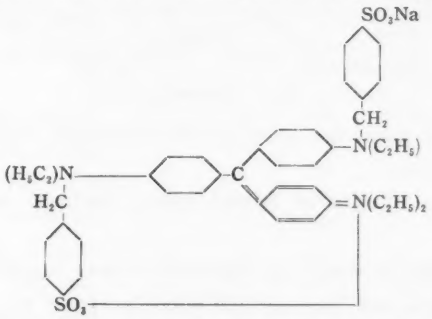
In 1956, I (1) reported that several dyes (rhodamine B, rhodamine 6 G, fluorescein sodium, eosine yellowish, acridine red (2) and toluyleneblue (3)) were carcinogenic to experimental animals when repeatedly injected into the same site for a long period of time.

In the present paper are first given details of the similar injection experiments in which certain substances failed to produce sarcoma (Exps. I-IV; acid violet, auramine, sodium salicylate and 8-oxyquinoline sulfate). Feeding experiments with negative results of two substances (auramine G and salicylic acid) are next taken up (Exps. V and VI). These negative results are considered to be of some practical value since the substances tested are all food additives.

Injections of Acid Violet, Auramine, Sodium Salicylate and 8-Oxyquinoline Sulfate

These substances are connected with the human life through their use as food additives in modern methods of food production and processing.

Experiment I. Acid violet was tested in this experiment. Acid violet (Colour Index No. 698; $C_{41}H_{44}N_3O_6S_2Na$) is 4-(4-(N-ethyl-p-sulfobenzylamino)-phenyl)-(4-(N-ethyl-p-sulfonium benzylamino)-pheny)-methylene)-N, N-dimethyl-2, 5-cyclohexadienimine (monosodium salt), forming violet powder, the watery solution of which has violet colour. The dye is used for coloring such food products as chocolate, candies, jellies, frozen desserts, baberages, icings, puddings, etc.



The preparation of acid violet used in the experiment was a product of the

Tokushu Chemical Co., Ltd., Tokyo.

Experiment under the usual conditions was started with 16 normal rats of a mixed strain from the Saitama Prefecture, all weighing around 180 g.

Injections were made subcutaneously on the back of the rats at as nearly the same site as possible every time. The injection of 1 cc of 5.0 gdl distilled water solution of acid violet was repeated once every month, and after about 3 months, the dye solution was reduced to 0.5 cc. After about 9 months of repeated injections, the injections were discontinued.

8 rats survived 200 days or more, the longest survival period being 426 days (Table 1).

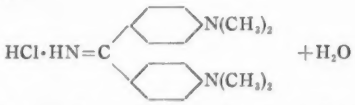
None of the animals developed tumor at the site of the injection. However, in the course of the experiment, one of them, dying on the 359th day, showed hypertrophy of thymus (2.0×1.4×1.2 cm), and another killed on the 426th day, had a cysticercus sarcoma of the liver. These tumors may be regarded as of the spontaneous origin, not connected with the experimental effect.

No significant change was noted in the local subcutaneous tissue as well as in internal organs at autopsy. The local tissues about the site of injection soon became deeply stained, and some violet color persisted for many months after the cessation of the injection.

Table 1. Experiment I.

Rat No.	Sex	Exp. days	Acid Violet		Final Body Weight (g)
			Total (mg)	Inject. No.	
1	/	204	125	3	/
2	f	258	200	6	/
3	m	261	200	6	/
4	f	342	225	7	/
5	m	349	225	7	/
6	m	354	225	7	/
7	f	359	225	7	270
8	m	426	225	7	165

Experiment II. Auramine (Colour Index No. 655; $C_{17}H_{22}N_3Cl+H_2O$) is hydrochloride of tetramethyldiamino-diphenyl-ketonimine, forming sulphur-yellow powder.



The preparation of auramine used in the experiment was the product of the Merck, Germany.

Animals used were 10 rats of hybrid strain (Saitama strain), all weighing around 150 g.

Auramine is insoluble in water and therefore it was suspended in water at the concentration of 0.5 gdl, and was injected subcutaneously on the back of the rats in 1 cc amounts, delivered into as nearly the same site as possible once or twice

weekly as a rule, and after 20 days, the dye solution was increased to 2 cc. 5 rats survived 200 days or more, one of the highest longevity surviving 463 days (Table 2).

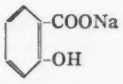
Table 2. Experiment II.

Rat No.	Sex	Exp. days	Auramine	
			Total (mg)	Inject. No.
1	/	216	185	37
2	f	358	225	45
3	f	421	240	48
4	f	440	245	49
5	f	463	245	49

None of the animals developed tumor at the site of the injection. However, in the course of the experiment, one of them, dying on the 440 th day, showed a mammary fibroadenoma (3.2×2.5×1.1 cm), presumably of the spontaneous origin.

Findings at autopsy: the local tissue at the site of the injections showed a fibrosis and hyaline degeneration. The liver was generally atrophic and histologically showed increase of Kupffer's stellate cells. Spleen showed some fibrosis.

Experiment III. Sodium salicylate (HOC_6H_4COONa) was tested in this experiment.



The preparation of sodium salicylate used in the experiment was the product of the Iwaki Seiyaku Co., Ltd., Tokyo.

Experiment was started with 25 normal adult albino rats of a mixed Saitama strain, all weighing around 150 g. The rats were given subcutaneous injections, at as nearly the same site as possible on the back, of 2 cc of 1.25 gdl waterly solution of sodium salicylate once every week as a rule.

9 rats survived 300 days or more, one of the highest longevity surviving 550 days (Table 3).

Table 3. Experiment III.

Rat No.	Sex	Exp. days	Sodium Salicylate		Final Body Weight(g)
			Total (g)	Inject. No.	
1	m	305	0.825	32	/
2	m	370	1.0	40	220
3	m	378	1.025	41	195
4	m	391	1.0	39	/
5	m	395	1.0	39	/
6	m	460	1.1	43	/
7	m	495	1.225	48	/
8	m	500	1.5	59	210
9	m	550	1.4	55	240

None of the animals developed tumor at the site of the injection.

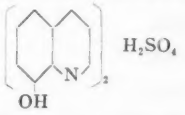
In the course of the experiment, two of the rats, dying on 294th and 391st days respectively, had a cysticercus sarcoma (spindle cell sarcoma) of the liver, and two others, dying on 460th and 495th days, showed hyper-

trophy of thymus (2.6×1.5×1.2 and 2.5×1.7×1.7 cm). Also one other, killed on 500th day, showed hypertrophy of the hypophysis (small pea size). All these tumors may be regarded as of the spontaneous origin.

The local tissue change was chiefly fibrosis. The liver was generally atrophic.

Intratesticular Injections of 8-Oxyquinoline Sulfate in Hamsters

Experiment IV. 8-Oxyquinoline sulfate which is used as a contraceptive agent was tested in this experiment, injecting it intratesticulary in hamsters.



The preparation of 8-oxyquinoline sulfate used in the experiment was the product of the Takeda Chemical Co., Ltd., Tokyo. Injections were made into the right testicle of the hamster. The injection of 0.05 cc of 10 gdl distilled water solution of 8-oxyquinoline sulfate, sterillized by heating before injection, was repeated once or twice weekly as a rule. Experiment was started with 12 normal hamsters, of which 5 survived 200 days or more, one of the highest longevity surviving 504 days (Table 4).

Table 4. Experiment IV.

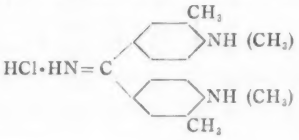
Hamster No.	Sex	Exp. days	8-oxyquinoline sulfate	
			Total (mg)	Inject. No.
1	m	204	370	47
2	m	348	450	55
3	m	372	450	55
4	m	377	450	55
5	m	504	450	55

None of the animals developed tumor at the site of the injection.

The injected testicle was generally atrophic, and showed degeneration in the cases of Nos. 1 and 3. The liver was congested and showed hemosiderosis. Sometimes, in later stages, degeneration or necrosis of liver cells was noted. Spleen showed congestion, atrophy and hemosiderosis.

Auramine G Feeding in Mice

Experiment V. Auramine G (Colour Index No. 656; $C_{17}H_{22}N_3Cl$) is a hydrochloride of dimethyldiaminoditolyl-ketonimine, forming yellow powder. The substance is said to be used in Japan for coloring various food articles.



The preparation of auramine G used in the experiment was the product of the Hodogaya Chemical Co., Ltd., Tokyo.

Experiment was started with 40 normal mice (both sexes) of a mixed Saitama strain, all weighing around 20 g. They were maintained on rice diet, to which auramine G was mixed evenly at the rate of 0.15%. The diet was supplemented with occasional supply of dried fish, cod-liver oil and green vegetables. The feeding of the dye was occasionally interrupted because of the toxic effect.

Table 5. Experiment V.

Mouse No.	Sex	Exp. days	Auramine G (mg)
1	m	156	228
2	m	156	228
3	f	159	234
4	m	180	288
5	m	260	474
6	f	316	598
7	m	352	679
8	f	359	694
9	m	369	718

9 mice survived 150 days or more, one of the highest longevity surviving 369 days (Table 5).

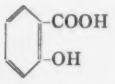
None of the animals developed a tumor in any organ in the course of the experiment.

The liver showed degeneration of liver cells and increase of Kupffer's stellate cells. Spleen showed congestion, atrophy, fibrosis and increase of spleen cells.

Salicylic Acid Feeding in Rats

The purpose of this experimental was to ascertain whether the feeding of salicylic acid, known to produce mutation, would cause pathological changes, especially malignant tumors, when continued for a long period of time.

Experiment VI. The preparation of salicylic acid used in the experiment was the product of the Koso Chemical Co., Ltd., Tokyo.



Experiment was started with 34 normal rats of a mixed Saitama strain, all weighing around 200 g. They were maintained on rice diet, to which salicylic acid ($C_6H_4(OH)CO_2H$) was mixed evenly at the rats of 0.5–1.0 per cent. The diet was occasionally supplemented with dried fish, cod-liver oil, olive oil and green vegetables. The amount of salicylic acid added was 5 g per 1 kg of rice for about 8 months, increased to 10 g per 1 kg for the rest of the experimental period. The feeding of salicylic acid was continued without interruption.

9 rats survived 200 days or more, one of the highest longevity surviving 331 days (Table 6).

Table 6. Experiment VI.

Rat No.	Sex	Exp. days	Salicylic Acid Total (g)
1	f	230	14.25
2	f	246	19.0
3	f	261	17.25
4	f	270	21.4
5	/	277	22.1
6	m	317	18.9
7	f	320	20.4
8	f	322	20.6
9	f	331	21.5

None of the animals developed tumor in any organ in the course of the experiment.

The stomachs of the experimental rats showed fibrosis and ulcer in a single case, dying on the 161st day (Total salicylic acid: 11.69 g). The liver showed cloudy hypertrophy. Spleen showed fibrosis, hemosiderosis and atrophy.

Discussion

Miller and Pybus (4) reported that leukemia was produced by acid violet in the mouse. Yao (5) fed auramine mixed with the usual laboratory diet to rats, but he failed to find tumor. Carcinogenic activity of salicylic acid has been tested in the past by Hosino (6), but no indisputable malignant tumor was produced. In my experiments none of these substances showed any indication of carcinogenic action.

Attention may be called to the problems of coloring matters for various food articles and food additives as potential carcinogenic hazards which have sprung up in the world lately. Hueper (8), Truhaut (9), etc., have discussed these problems, and symposia on potential cancer hazards from chemical additives to foodstuffs took place in Bad Godesberg in 1954 (7) and again in Rome (1956) under the sponsorship of the International Union against Cancer.

In view of these facts, the negative data presented in this paper would seem to be of sufficient interest to be reported.

Summary

1. Under the conditions of the above described experiments, acid violet, auramine, sodium salicylate, 8-oxyquinoline sulfate, auramine G and salicylic acid did not show carcinogenic activity.

2. No marked change in internal organs was found in the rats, hamsters and mice under the conditions of the respective experiments. It may be noted,

however, that auramine produced some degeneration of liver cells in mice and salicylic acid a mild atrophy of spleen in rats.

Acknowledgements

I take pleasure in acknowledging my indebtedness to Dr. Waro Nakahara and Dr. Makoto Tanaka for their help and encouragement in the course of this work.

References

(1) Umeda, M.: Experimental study of xanthene dyes as carcinogenic agents, Gann, **47**, 51-78, 1956.

(2) Umeda, M.: Sarcoma production by injections of acridine red. A supplement to experimental study of xanthene dyes as carcinogenic agents, Gann, **47**, 153-158, 1956.

(3) Umeda, M.: Experimental carcinogenesis: Rat rhabdomyosarcoma produced by the injection of toluylene blue, Gann, **45**, 447-449, 1954.

(4) Miller and Pybus: Brit. Cancer Campaign, **32**, 246, 1955.

(5) Yao, M.: Osaka Igaku Zasshi, **36**, 1485, 1937.

(6) Hosino, I.: Zikken Syokaki Byo Gak. Zassi, **15**, 5-49, 1940.

(7) Summary of a meeting of West European Scientists on the prophylaxis of cancer. 1954.

(8) Hueper, W. C.: Potential role of non-nutritive food additives and contaminants as environmental carcinogens, A. M. A. Arch. Path., **62**, 218-249, 1956.

(9) Truhaut, R.: Le problème des substances étrangères dans les aliments du point de vue cancerologique, Oncologia, **9**, 84-134, 1956.

要　旨

食品添加用諸化学物質の発癌実験（第 1 報）

梅田真男（癌研究所）

1956 年にローマで癌の国際会議が開催され，食品，石鹸，化粧品の製造や保存に用いられている色素や添加物の発癌性についていろいろと討論され，この問題が各国において重要視されている現在，私は下記の 6 種類の食品添加物について，発癌性の問題を研究した。

物　質　名	投　与　法	実験動物	最高生存日数	癌発生数
Acid Violet	皮下注射	ダイコクネズミ	426 日	0
Auramine	皮下注射	ダイコクネズミ	463 日	0
Sodium Salicylate	皮下注射	ダイコクネズミ	550 日	0
8-Oxyquinoline Sulfate	睾丸注射	ハムスター	504 日	0
Auramine G	経　口	ハツカネズミ	369 日	0
Salicylic Acid	経　口	ダイコクネズミ	331 日	0

以上の表の如く，アシッドバイオレット，オーラミン，サリチル酸ソーダ，8-オキシキノリン硫酸塩の注射実験，及びオーラミン G，サリチル酸の経口投与実験では，動物に癌はできなかった。しかしオーラミン G は肝臓に変性を，またサリチル酸は脾臓の萎縮硬化を起した。現在種々の食品添加物について研究中である。これらの研究が癌と食生活の問題への寄与となれば幸と思う。

（厚生科学研究費による）

[GANN, 48, 65~72; March, 1957]

GLYCOLYTIC INHIBITION OF CARCINOSTATIC QUINONE AND QUINOLINE DERIVATIVES

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and SUMIO SAKAI
(Laboratories of the Kaken Chemicals, Ltd., Tokyo)

In previous papers (1, 2) we reported that certain quinone and quinoline derivatives show a distinct anti-cancer action as evidenced by a significant prolongation of the period of survival of mice after the intraperitoneal inoculation of Ehrlich ascites carcinoma. Among the quinone derivatives, 2-methylthio-1,4-naphthoquinone gave perhaps the best result, while among the quinoline derivatives 4-nitroquinoline-N-oxide, 6-bromo-4-nitroquinoline-N-oxide and 2-ethyl-4-nitroquinoline-N-oxide stood out as even more effective. In spite of the fact that the testing of cancerocidal action *in vitro* is regarded as of little value by some authorities, it is noteworthy that these effective quinone and quinoline derivatives were found as the result of preliminary *in vitro* screening tests (Fukuoka's method) (3), which has been extensively employed in our laboratory.

At the present time, the mode of action of the quinone and quinoline derivatives with potent anti-cancer activity remains unclarified. In order to amend this situation we describe in this paper our recent experiments which demonstrate a remarkable parallelism which is shown by these derivatives between the anti-cancer action and the inhibiting effect on anaerobic glycolysis of Ehrlich carcinoma cell suspensions, measured by the ordinary manometric method using Warburg's apparatus.

The essential point in this study consists in discovering how the data on anti-cancer activity fit into those of the glycolysis inhibiting effect. For this purpose we used our previous data on the cancerocidal action *in vitro*, which covered much wider range of derivatives than our *in vivo* experiments. We believe that at least in dealing with quinone and quinoline derivatives there is a fair correlation between the results of *in vitro* and *in vivo* effects, which justifies the use of the *in vitro* data as representing the over-all anti-cancer activity of the derivatives.

METHODS

The quinone and quinoline derivatives to be tested were selected from a large

series of these substances already tested as to their anti-cancer activities, which have been reported in full in two previous papers (1, 2). In making the selection care was taken to include representatives, not only of groups with strong, moderate and practically no tumoricidal activities, but also of different chemical groups as indicated by their structures.

The procedures of measuring glycolysis were as follows:—Ascites fluid of Ehrlich mouse carcinoma was aspirated one week after inoculation. It was at once diluted with about five volumes of cold physiological saline and contaminating red cells were removed by low speed centrifugation as described by McKee *et al.* (4). Finally, cells were suspended in Krebs-Ringer-bicarbonate buffer solution at suitable concentration. In each Warburg vessel was placed a total of 3 ml of solution, containing glucose in the final concentration of 200 mg/dl, and $1.0\text{-}1.6\times10^7$ cells. The gas phase was 95% nitrogen and 5% carbon dioxide. After 10 minutes' temperature equilibrium time, the evolved carbon dioxide coused by lactic acid formation in bicarbonate buffer was measured at 10 minutes intervals for one hour. The substance to be tested was dissolved in Krebs-Ringer-bicarbonate solution with the aid of a very small amount of propyrene glycol. The final concentration of propylene glycol in vessels was 1.3 per cent. Control vessels contained propylene glycol at the same concentration.

Results

The results obtained from the experiments of quinone derivatives are represented in Figure 1. 2-Methyl-3-hydroxy-1,4-naphthoquinone having no tumoricidal action, does not inhibit anaerobic glycolysis at all. Four other derivatives having

Table 1. Effect of Quinone Derivatives on the Glycolysis of Ehrlich Ascites Carcinoma Cells and Tumoricidal Effect *in vitro* of N F Mouse Sarcoma (1).

No.	Substance	Glycolytic Inhibition		Tumoricidal Effect* at		
		30 min	60 min	0.05	0.01	0.005%
1883	2-Methylthio-1, 4-naphthoquinone	98.2%	98.8%	6/6	6/6	5/6
1886	2, 3-Dimethylthio-1, 4-naphthoquinone	95.3	96.8	6/6	6/6	3/6
1913	2-Methyl-1, 4-naphthoquinone	94.7	96.5	4/4	3/4	3/4
1981	2-Methyl-3-hydroxy-1, 4-naphthoquinone	0	0	—	—	—
1991	1, 4-Naphthoquinone oxime	44.2	43.5	3/3	3/3	2/3

*Tumoricidal effect in Tables 1 and 2 are expressed in the team of the number of negative over the total number of implants for each dilution of test substances. For example 3/3 means that all the implants resulted in no tumor growth, 1/3 only one out of three implants resulted in negative, i.e., two tumors obtained from three implants. The case where all implants produced tumors are indicated by minus signs (—), meaning that there was no effect.

Table 2. Effect of Quinoline Derivatives on the Glycolysis of Ehrlich Ascites Carcinoma Cells and Tumoricidal Effect *in vitro* of NF Mouse Sarcoma (2)

	No.	Substance	Glycolytic Inhibition		Tumoricidal Effect* at					
			30 min	60 min	0.05	0.01	0.005	0.002	0.001	0.0005%
Quinoline-N-oxide	1935	Quinoline-N-oxide dihydrate	1.8%	1.2%	—	—	—	—		
	2055	Quinaldine-N-oxide	0	5.0	—	—	—	—		
	2124	3-Methylquinoline-N-oxide	0	0	—	—	—	—		
	2144	7-Chloroquinoline-N-oxide	1.7	0	—	—	—	—		
	2067	6 Bromoquinoline-N-oxide	4.4	19.2	—	—	—	—		
Nitroquinoline	2052	4-Nitroquinoline	6.7	8.3	—	—	—	—		
	3030	6-Nitroquinoline	13.7	7.2	—	—	—	—		
	2045	8-Ethoxy-5,7-dinitroquinoline	30.0	47.2	3/3	1/3	—	—		
	2020	5-Nitroquinaldinic acid	0	0	—	—	—	—		
	2446	5-Nitroso-8-hydroxyquinoline hydrochloride	4.6	8.0	2/2	—	—	—		
	2656	2-Methyl-8-hydroxyquinoline	0	0	—	—	—	—		
4-Nitroquinoline-N-oxide	1936	4-Nitroquinoline-N-oxide	31.4	37.2	4/6	6/6	4/6	3/3	±	—
	2054	4-Nitroquinaldine-N-oxide	74.1	79.3	4/4	4/4	4/4	3/3	±	—
	2126	4-Nitro-2-ethylquinoline-N-oxide	83.6	87.3	3/3	3/3	3/3	3/3	3/3	3/3
	2068	6-Bromo-4-nitroquinoline-N-oxide	77.3	78.6	1/3	±	±			
	2149	6-Chloro-4-nitroquinoline-N-oxide	72.8	73.0	3/3		2/3	2/2	2/2	±
	2147	7-Chloro-4-nitroquinoline-N-oxide	55.0	66.3	3/3		±			

* Tumoricidal effect in Tables 1 and 2 are expressed in the team of the number of negative over the total number of implants for each dilution of test substances. For example 3/3 means that all the implants resulted in no tumor growth, 1/3 only one out of three implants resulted in negative, i.e., two tumors obtained from three implants. The case where all implants produced tumors are indicated by minus signs, (—), meaning that there was no effect. Case in which there were only small tumors compared to the control group are indicated by the ± sign.

Figure 1

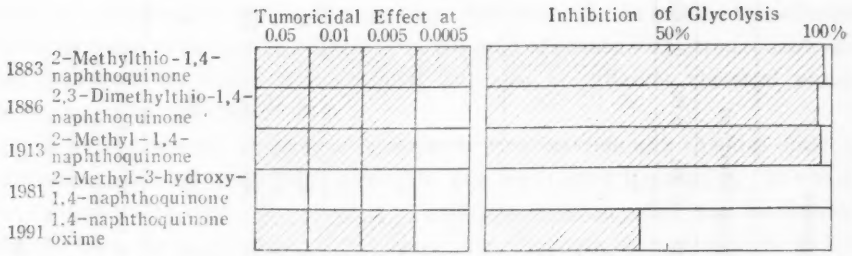
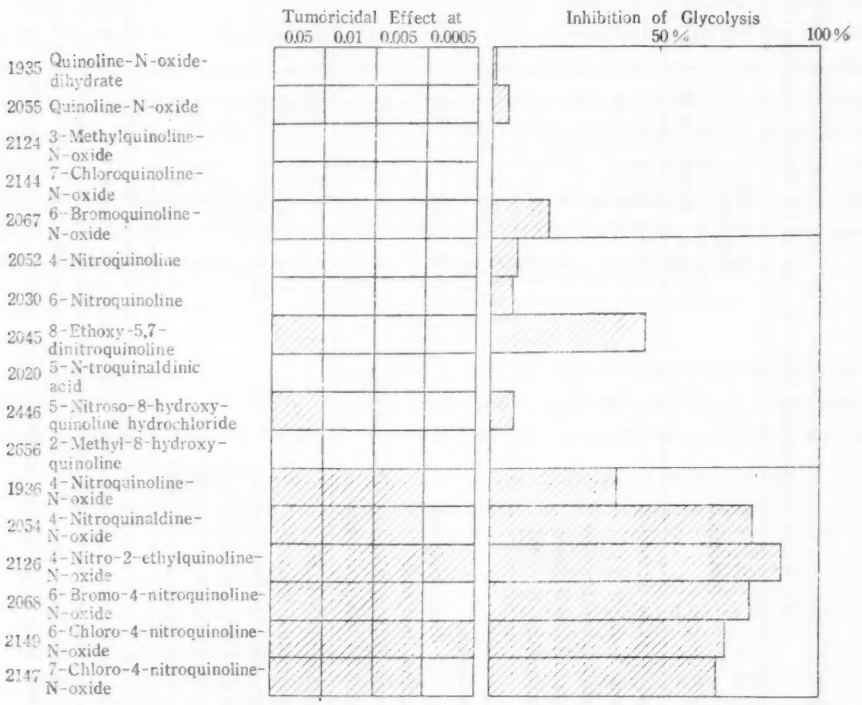


Figure 2.



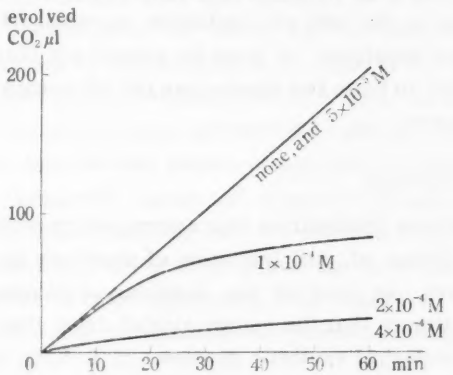
tumoricidal action, strongly inhibit anaerobic glycolysis. The full data are given in Table 1. In this experiment, the final concentration of the substances tested is 4×10^{-4}M.

The results of experiments using quinoline derivatives are given in Figure 2. The upper group is quinoline-N-oxide derivatives, and middle and lower groups are nitroquinoline and 4-nitroquinoline-N-oxide derivatives, respectively. The first group, quinoline-N-oxide derivatives, has neither tumoricidal action nor inhibit-

ing action on anaerobic glycolysis. Among the second group, nitroquinoline, 8-ethoxy-5, 7-dinitroquinoline which has a slight tumoricidal action, shows moderate inhibiting action on anaerobic glycolysis. As an exceptional case, 5-nitroso-8-hydroxyquinoline hydrochloride, with a moderate tumoricidal action, shows only slight inhibition on anerobic glycolysis. The third group, namely, 4-nitroquinoline-N-oxide, shows strong tumoricidal and glycolysis-inhibiting actions. The full data are furnished in Table 2. The final concentration of the substances tested is 4×10^{-4}M.

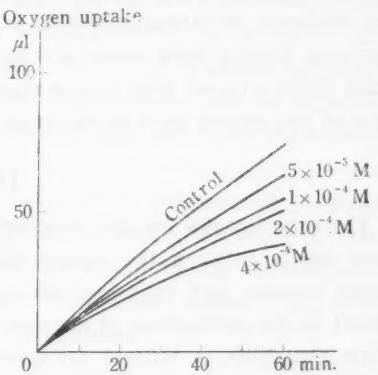
The inhibition of anaerobic glycolysis in Ehrlich carcinoma cell suspension at various concentrations of 4-nitro-2-ethylquinoline-N-oxide is illustrated in Figure 3. This substance has no inhibiting action at the concentration of 5×10^{-5}M but it causes a remarkable inhibition at the concentration of 2×10^{-4}M. When the concentration exceeds this level, no further increase of inhibiting action is observed. This phenomenon is not yet explained.

Figure 3. Inhibition of Glycolysis of Ehrlich Carcinoma Cells by 4-Nitro-2-ethylquinoline-N-Oxide at Different Concentrations



3 ml of Krebe-Ringer-Bicarbonate Solution containing 1.6×10^7 cells in each vessel. Glucose concentration, final 200mg/dl

Figure 4. Inhibition of Respiration of Ehrlich Carcinoma Cells by 4-Nitro-2-ethylquinoline-N-Oxide



3 ml of Krebs-Ringer-Phosphate Solution containg 2.5×10^7 cells in each vessel. No addition of substrate.

Figure 4 shows the inhibition of respiration of Ehrlich carcinoma cell suspension at various concentrations of 4-nitro-2-ethylquinoline-N-oxide. It is obvious from the comparison of Figure 3 with Figure 4 that, with this substance, the inhibition of anaerobic glycolysis occurs at a lower concentration than that of respiration.

In addition to quinone and quinoline derivatives, we tested thio-TEPA (TESPA), but here the inhibition of anaerobic glycolysis at the same concentration of 4×10^{-4}M was only 3.5 per cent in one hour.

Table 3. Glycolytic Inhibition in Various Tumors and Normal Spleen by 2-Methylthio-1,4-naphthoquinone (1883) and 4-Nitro-2-ethylquinoline-N-oxide (2126).

	1883		2126	
	30 min	60 min	30 min	60 min
Cell suspension of Ehrlich carcinoma	98.2%	98.8%	83.6%	87.3%
Cell suspension of rat hepatoma (130)	62.5	66.3	72.9	77.5
Slice of solid Ehrlich carcinoma	38.5	52.0	41.5	37.4
Slice of N F sarcoma	55.0	65.0	46.0	64.9
Slice of rhodamine sarcoma	14.5	17.5	38.7	48.2
Slice of spleen	6.0	12.0	14.8	33.7

In Table 3 are shown results of experiments using the slices of normal rat spleen, rhodamine sarcoma (fibrosarcoma, rat), NF sarcoma (fibrosarcoma, mouse) and solid tumor of Ehrlich carcinoma and the suspension of transplantable ascites hepatoma (Yoshida's strain 130). 4-Nitro-2-ethylquinoline-N-oxide and 2-methylthio-1,4-naphthoquinone are used at the same concentration of 4×10^{-4}M. Both of these agents show a slight inhibiting action in the case of normal spleen but moderate or strong inhibiting action in the case of rhodamine sarcoma, NF sarcoma, Ehrlich solid tumor and ascites hepatoma. It must be pointed out that solid Ehrlich tumor slice is less sensitive to these two agents than the cell suspension of the ascites form of the same tumor.

Discussion

The experimental results described above demonstrate that a very close parallelism exists between the tumoricidal action of, and inhibition of glycolysis by, some quinone and quinoline derivatives. In view of the numerous evidences found in the metabolism of neoplastic tissues that the energy yielded from their high glycolysis is utilized for many biological synthetic processes (5, 6, 7, 8), it would follow that the above results are strongly suggestive of the mode of action of these tumoricidal substances.

The affinity of 2-methyl-1,4-naphthoquinone to sulfhydryl group is described (9), and the binding of 4-nitroquinoline-N-oxide with sulfhydryl groups is reported (10). So there is a possibility that 3-phosphoglyceraldehyde dehydrogenase, which is well known as a sulfhydryl enzyme and is noticeably blocked by monoiodoacetic acid, may be inhibited by these quinone and quinoline derivatives. Naturally, the blocking of sulfhydryl group of other enzymes and metabolites essential for growth must also be considered, but the fact that the glycolytic inhibition occurs at a lower concentration than respiratory inhibition, would suggest the important role of the former in tumoricidal action. The same pheno-

menon is recently reported by Holzer *et al.* in the action of ethyleneimine compounds on Ehrlich carcinoma cells (11). Our data also completely agree with the report that monoiodoacetic acid inhibits glycolysis at the low concentration, which does not produce any inhibition on respiration, and that Ehrlich carcinoma cells treated with monoiodoacetic acid *in vitro* show the retardation of growth after implantation in mice (12).

The fact that, in our experiments, thio-TEPA (TESPA) was proved to have no glycolysis inhibiting action at the comparable concentration would suggest, too, the importance of glycolytic inhibition in the action of quinone and of quinoline. It was reported that TESPA at the concentration of 1.9×10^{-3}M on Ehrlich carcinoma cells (11, 13) and TEM at 1.0×10^{-3}M on the ascites form of Krebs II carcinoma (14) did not cause any inhibition of glycolysis. In consideration of these data, it can be concluded that the carcinocidal quinone and quinoline derivatives have a strong affinity to glycolytic systems at a considerable low concentration.

The fact that inhibition of glycolysis is very slight in normal spleen appears to us to be fascinating. But, since the slices of solid Ehrlich tumor and of other tumor tissues were less inhibited by these agents than Ehrlich carcinoma ascites cell suspension, the above fact can probably be explained by the failure of permeation of these agents in the case of the slices. In the case of ascites hepatoma which forms cell-islands in ascitic fluid, the degree of permeation may be expected to be intermediate between the cases of free cell and slice, and it actually showed the intermediate degree of glycolytic inhibition.

Mutagentic action of 2-methyl-1,4-naphthoquinone on *Micrococcus pyogenes* (15) and *E. coli* (16) and of 4-nitroquinoline-N-oxide on *Aspergillus niger* (17) are reported. We must consider the investigation pertaining to the radiomimetic action of these substances with regard to their tumoricidal action. This is a problem to be elucidated in future by further experiments.

Summary

Selected representatives of quinone and quinoline derivatives were tested as to their effect on the anaerobic glycolysis of Ehrlich carcinoma cell suspension, and a close parallelism was noted between the glycolysis-inhibiting effect and the anti-cancer action, observed in previous experiments, of the respective derivatives. The significance of, and implications from this parallelism were discussed.

References

1. Sakai, S., Minoda, K., Saito, G., and Fukuoka, F.: Gann, **46**, 59 (1955)
2. Sakai, S., Minoda, K., Saito, G., Akagi, S., and Fukuoka, F.: Gann, **46**, 605 (1955)
3. Fukuoka, F.: J. Sci. Res. Inst., **29**, 491 (1953)

4. McKee, R. W., Lonberg-Holms, K., and Joann, J.: Cancer Res., **13**, 537 (1953)
5. Clowes, G. H. A., and Keltch, A. K.: Proc. Soc. Exptl. Biol. Med., **81**, 356 (1952)
6. LePage, G. A., Potter, V. R., Bush, H., Heidelberger, C., and Hurlbert, R. E.: Cancer Res., **12**, 153 (1952)
7. LePage, G. A.: Cancer Res., **13**, 178 (1953)
8. Rabinovitz, M., Olson, M. E., and Greenberg, O. M.: J. Biol. Chem., **213**, 1 (1955)
9. Fieser, L. H., and Fieser, M.: "Organic Chemistry" D. C. Heath and Co., Boston, 1944
10. Okabayashi, T.: J. Pharmaceutical Soc. Japan, **73**, 946 (1953)
11. Holzer, H., Sedlmayer, G., and Kemnitz, A.: Biochem. Z., **328**, 163 (1956)
12. Holzer, H., Haan, J., and Pette, D.: Biochem., **327**, 195 (1955)
13. Ebina, T., and Kurosu, M.: Gann, **47**, 247 (1950)
14. Roitt, I. M.: Biochem. J., **63**, 300 (1956)
15. Clark, B., Wyss, C., and Stone, W. S.: Nature, **166**, 340 (1950)
16. Colwell, C. A., and McCall, M.: J. Bacteriol., **51**, 659 (1946)
17. Okabayashi, T.: J. Fermentation Technol., **33**, 513 (1955)

要　旨

抗癌性キノン及びキノリン誘導体による解糖阻害

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抗癌性を有するキノン，キノリン誘導体の中代表的なものをえらび，エーリッヒ腹水癌細胞の嫌気的解糖におよぼす影響をしらべた。その結果，抗癌性の強さと，解糖阻害の強さとの間に，密接な平行関係のある事実を見出すことができた。これらの結果を記載した。

(文部省科学研究費による)

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FERRITIN CONTENT OF THE LIVER OF TUMOR-BEARING ANIMALS

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It is well established that the low liver catalase activity and anemia are pronounced in tumor-bearing animals. In view of the inhibition of the synthesis of catalase molecules in tumor-bearing animals, it is important to ascertain whether or not porphyrin, protein and/or iron metabolisms are disturbed at the same time. As one of a series of such studies (1, 2, 3), ferritin content of the liver of animals bearing growing neoplastic tissues was determined. Ferritin has many physiological activities (4) and iron in the form of colloidal ferric-hydroxide-phosphate-micells accounts for 25 per cent of its composition. Ferritin plays an important role in the storage and metabolism of iron in the animal body (5, 6).

Material and Methods

For the sake of simplicity and to secure the homogeneity of experimental conditions, the experiments were done on mice, which were maintained on the identical diet. Transplantable NF mouse sarcoma (fibrosarcoma) and NF carcinoma (adenocarcinoma) were used. About three weeks after transplantation, when tumors grew to at least about 3 g, animals were killed and the liver catalase activity was determined by the method of Euler and Josephson (7) and the ferritin content by the method described by Yoneyama and Konno, which was based on the characteristic fact that ferritin in physiological saline homogenate was not precipitated by heating at 80°C but was at 100°C (8). The precipitate, which was formed between 80°C and 100°C on heating physiological saline homogenate of tissues, was extracted twice with the mixture of the equal volume of 10 per cent trichloracetic acid and 4 per cent sodium pyrophosphate at 100°C for 15 minutes. The iron content of extract was determined by the color reaction after the addition of thioglycolic acid and o-phenathroline. According to the original authors the value of ferritin content estimated by this procedure, which depends on the iron determination of this fraction, completely agreed with the value obtained by the immunochemical procedure.

Results and Comments

The results are tabulated in Table 1. No decrease whatever of liver ferritin content was recognized in experimental animals, in spite of the fact that in the

very same animals the decreased liver catalase activity was found without exception. It was only in two animals having the erosion on the surface of tumor that decreased ferritin content was observed.

Table 1. Ferritin Content of Liver of Normal and Tumor-bearing Animals

Animal No.	Sex	Transplanted Tumor	Body Weight	Tumor Weight	Days after Transpl.	Ferritin Iron	Liver Catalase Activity	Notes
Tumor-bearing			g	g	days	μg/g	*k*/10 mg	
1.	m	NF sarcoma	17.5	4.5	25	36.6	—	
2.	m	NF carcinoma	18.3	3.6	23	34.2	—	
3.	m	NF carcinoma	11.9	2.3	20	56.7	0.1445	
4.	m	NF carcinoma	14.0	3.3	20	44.8	0.0943	
5.	m	NF sarcoma	15.5	3.2	20	33.8	0.0598	
6.	m	NF sarcoma	13.9	5.1	20	3.2	0.0502	Tumor with erosion
7.	m	NF sarcoma	13.2	7.0	20	43.5	0.1070	
8.	m	NF carcinoma	15.6	4.4	18	35.7	0.1070	
9.	f	NF sarcoma	17.6	6.4	23	28.4	0.1505	
10.	m	NF sarcoma	17.3	9.2	23	4.1	0.1135	Tumor with erosion
Normal								
1.	m		18.5			42.1	—	
2.	m		18.0			24.2	—	
3.	m		18.7			37.3	—	
4.	m		19.0			18.7	0.4820	
5.	m		18.0			28.4	0.5360	
6.	m		18.0			39.1	0.5560	
7.	m		18.0			24.8	0.2235	
8.	m		20.0			40.3	0.3105	
9.	m		17.0			40.1	0.2704	

Recently, Iijima *et al.* (9) and Sasori (10) reported a decreased ferritin content of the liver of gastric cancer patients. It will be solved in future whether decrease in their cases is specific to gastric cancer or not. Theorell *et al.* (11) observed that the appearance of the specific activity of liver ferritin preceded that of catalase when radioactive iron was injected into normal guinea pigs. The iron of ferritin was utilized after reduction to ferrous iron. From the fact that the liver ferritin content did not decrease in tumor-bearing animals, it is suspected that the iron absorption from intestinal canal and its storage is not greatly impaired. On the other hand, the mechanism of iron incorporation into porphyrin seems to be disturbed in some way. It must be pointed out that free porpyrin increased in liver, erythrocyte and urine of tumor-bearing animals as described in our

previous papers (2, 3). This fact suggests that the metabolic impairment in these animals involves a deficient utilization of porphyrin. The decrease of ferritin content in animals which have erosion on the surface of tumor may be interpreted as the results of bleeding (6, 10), and there is a possibility that the same interpretation may apply to the recent findings of Iijima *et al.* (9) and Sasori (10) already referred to.

References

1. Fukuoka, F., and Nakahara, W.: Gann, **42**, 55 (1951).
2. Sugimura, T., Umeda, M., and Ono, T.: Gann, **47**, 87 (1956).
3. Ono, T., Umeda, M., and Sugimura, T.: Gann, **47**, 171 (1956).
4. Granick, S.: Phsyiological Review, **31**, 489 (1951).
5. Shorr, E.: The Harvey Lecture, **50**, 112 (1954-1955).
6. Shoden, A., Gabrio, B.W., and Finch, C. A.: J. Biol. Chem., **204**, 823 (1953).
7. Euler, H., and Josephson, K.: Ann., **452**, 58 (1927).
8. Yoneyama, M., and Konno, K.: J. Biochem., **40**, 377 (1953).
9. Iijima, N., Matsuura, K., and Fujita, K.: Gann, **47**, 267 (1956).
10. Sasori, T.: Gann, **47**, 294 (1956).
11. Theorell, H., Benzak, N., Bonickesen, R., Paul, K. G., and Akeson, A.: Acta Chem. Scand.: **5**, 443 (1951).

要　旨

担癌動物の肝フェリチン量

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担癌動物の肝タラーゼ活性低下，血液ヘモグロビン濃度低下等にかんがみ，その成り立ちに関する研究の一端として，肝フェリチン量を測定した結果，顕著に肝カタラーゼ活性の低下した動物肝でもフェリチン量にはほとんど変化のない事実を見出した。

(文部省科学研究費による)

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NON-PROTEIN AND PROTEIN SULFHYDRYL GROUPS IN NEOPLASTIC TISSUES

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Sulfhydryl groups have such important roles in various metabolisms as, for example, the well known part that glutathione plays in the biological oxidoreduction, in activation of amino acids in transpeptidation and in many other biological processes (1). Moreover, newer evidences seem to point to a close relation of sulfhydryl group to the mechanism of cell division. To mention only a few of the more recent reports concerning this latter aspect Rapkine observed that intracellular sulfhydryl groups of Arbacia egg is temporarily decreased after fertilization and returned to the initial level prior to first cleavage (2), and Mazia and Dan suspected that the change of sulfhydryl to disulfide linkage might be associated with the mechanism of the aster formation in dividing cells (3).

For obvious reasons the sulfhydryl group in neoplastic cells formed the subject of active investigation in the past and some data on the glutathione content of neoplastic tissues were already published (4-8). In the light of technical development in this field, however, we consider it worth while to reinvestigate the subject by determining the contents of sulfhydryl and disulfide groups, non-protein (acid soluble) and protein, by means of the amperometry which has the high specificity to sulfhydryl groups (11). Furthermore, we determined enzymatically the content of coenzyme A which has, in the terminal of its molecule, sulfhydryl as the functional group (10). In view of the high water content of neoplastic tissues, RNA, DNA, protein, nitrogen, and dry weight were determined for the interpretation of our results. In consideration of the fact that the amount of free peptide is increased in the liver of tumor-bearing animals, we also determined the sulfhydryl group in the liver of such animals.

Material and Methods

Male albino rats (Saitama mixed strain) and albino mice were used. As tumor materials, transplantable hepatoma, rhodamine sarcoma or m-toluylen-diamine-induced sarcoma of rats, quinone carcinoma, fructose sarcoma or NF sarcoma of mice were used. When the tumor reached a considerable size, animals were killed by decapitation and tissues were homogenized with 9 volumes of cold water. For the determination of non-protein sulfhydryl group, 4 parts of 5 per cent sul-

Table 1. Non-prortein Sulfhydyl Group in Neoplastic Tissues

Animal No.	Sex	Body Weight g	Tumor	Tumor Weight g	Days after transpl.	Nonprotein Sulfhydryl*		RNA mg/g	DNA mg/g	Protein mg/g	Dry Weight mg/g
						Reduced mg/100g	Oxidized mg/100g				
Rat 1	m	85	Hepatoma	30	13	78.7	10.2	7.50	6.82	130	—
Rat 2	m	103	Hepatoma	9	16	75.6	—	8.25	4.76	94	213
Rat 3	m	183	Rhodamine sarcoma	12	20	115.0	20.3	8.77	5.26	92	210
Rat 4	m	165	〃	14	20	107.8	—	8.03	5.31	76	184
Rat 5	m	117	〃	38	27	100.4	—	6.83	6.23	90	180
Rat 6	m	120	〃	18	11	100.0	32.0	—	—	—	175
Rat 7	m	105	Heaptoma	14	21	78.5	2.5	—	—	—	170
Rat 8	m	101	m-Toluylenediamine induced sarcoma	22	25	90.9	—	6.40	7.88	92	167
Mouse 1	m	18	Quinone carcinoma	9.0	30	110.6	—	7.88	8.60	124	202
Mouse 2	m	24	〃	5.0	30	94.0	0.0	7.65	7.26	112	215
Mouse 3	m	18	Fructose sarcoma	3.0	19	50.8	0.0	9.75	9.20	106	200
Mouse 4	m	15	〃	3.2	19	47.1	21.2	9.01	9.75	108	199
Mouse 5	m	18	NF sarcoma	7.7	23	60.1	—	9.90	4.76	82	194
Mouse 6	m	17	〃	5.5	23	73.6	—	10.28	4.92	110	178
Mouse 7	m	19	Ehrlich carcinoma	3.6	11	36.9	—	8.23	7.93	107	209
Fowl 1	m	—	Rous sarcoma	—	14	78.3	—	3.62	1.68	62	105

* mg expressed as glutathione.

fosalicylic acid was added and the supernatant after centrifugation was used. For the determination of sulfhydryl groups of protein, whole homogenate was used (9). Amperometry was carried out by the procedure of Goldzieher *et al.* with a slight modification. Disulfide group was amperometrically determined after reduction to sulfhydryl form by the addition of 10 per cent sodium sulfite (10). Precipitate after centrifugation was washed with ethanol and DNA and RNA were analyzed by the method of Ogur and Rosen (12). Coenzyme A was enzymatically determined by the method of Kaplan *et al.* (11)

Results and Comments

The results are tabulated in Tables 1–4. It is certain that the concentration of non-protein (acid soluble) sulfhydryl groups, most of which consists of glutathione, is far lower in rat hepatoma than in the liver of normal and tumor-bearing rats. Non-protein sulfhydryl group contents of rhodamine sarcoma, m-toluylenediamine-induced sarcoma, quinone carcinoma, fructose sarcoma and Rous sarcoma were approximately equal to that of rat hepatoma. Thus, increase of sulfhydryl and disulfide groups in neoplastic tissues was not found. The amounts of sulfhydryl and disulfide contents of protein per gm wet tissues were appreciably smaller in

Table 2. Non-protein Sulfhydryl Group in the Liver of Normal and Tumor-bearing Animals

Animal No.		Sex	Body Weight g	Liver Weight g	Non-protein Sulfhydryl*		RNA	DNA	Protein	Dry Weight
					Reduced mg/100g	Oxidized mg/100g	mg/g		mg/g	mg/g
Normal Animals										
Rat	9	m	234	6.9	216.5	—	8.77	4.05	181	322
Rat	10	m	107	4.8	175.0	—	10.49	4.30	153	299
Rat	11	m	167	—	207.0	—	7.88	6.00	193	284
Rat	12	m	170	—	155.0	—	—	—	—	—
Mouse	8	m	20	1.1	250.5	—	11.08	4.75	189	309
Mouse	9	m	19	0.9	212.0	—	12.88	4.88	163	280
Tumor-bearing Animals										
Rat	1	m	85	4.6	176.8	0.0	12.00	5.75	180	—
Rat	2	m	103	4.6	245.0	—	12.75	3.00	152	279
Rat	3	m	188	9.5	250.5	—	9.53	3.01	122	294
Rat	4	m	165	8.9	267.0	—	10.13	3.13	150	313
Rat	5	m	117	5.9	303.0	—	11.22	3.83	165	287
Mouse	1	m	18	1.1	161.1	—	12.00	6.50	183	256
Mouse	2	m	24	1.6	219.5	—	12.38	5.20	180	284
Mouse	3	m	18	1.0	240.5	—	12.88	5.55	168	274
Mouse	4	m	15	0.9	193.5	4.7	11.22	6.50	168	294
Mouse	5	m	18	1.1	277.0	—	13.28	4.33	154	274
Mouse	7	m	19	1.4	257.0	—	12.38	4.93	157	278

* mg expressed as glutathione.

neoplastic tissue than in liver, but the difference was not so remarkable when the amounts of sulfhydryl and disulfid contents per 100mg of protein were compared. The amount of coenzyme A was very small. This phenomenon corresponds

Table 3. Protein Sulfhydryl Group in Liver and Neoplastic Tissues

Tissue	Protein Sulfhydryl		Protein	Dry Weight	Protein Sulfhydryl	
	Reduced μM/g	Oxidized μM/g	mg/g	mg/g	Reduced μM/100mg protein	Oxidized
Rat liver	24.4	54.3	179	310.5	13.6	30.3
Rat liver	24.2	—	163	283.5	14.8	—
Rat m-toluylene diamine sarcoma	9.5	—	78	167.0	12.2	—
Rat rhodamine sarcoma	10.7	29.7	79	175.2	13.5	37.6
Rat hepatoma	7.9	14.0	76	169.5	10.4	18.4

to the fact that respiratory activity is rather low for a relatively high glycolytic activity, since coenzyme A is necessary for the pyruvate oxidation via the citric acid cycle. A slight increase of hepatic non-protein sulfhydryl group in tumor-bearing animal was observed, but the physiological meaning of this increase is not yet known.

It may be conclusively stated that sulfhydryl and disulfide groups of non-protein and protein fractions are not increased in neoplastic tissues.

Table 4. Coenzyme A Content of Liver and Neoplastic Tissues.

	units/g
Rat liver	118.4 177.0 162.5
Rat hepatoma	12.7 9.0
Rat rhodamine sarcoma	19.9 16.5
Mouse liver	150.0
Mouse Ehrlich carcinoma	8.3 trace
Mouse NF sarcoma	17.3 13.5

Reference

1) Barron, E. S. G.: Adv. in Enzymol., **11**, 201 (1951).
2) Rapkine, L.: cf. Brachet, J.: Chemical Embryology, p. 171.
3) Mazia, D., and Dan, K.: Adv. in Med. and Biol. Physics, **4**, 70 (1955).
4) Yaoi, H., and Nakahara, W.: Gann, **20**, 51 (1926), Biochem. J., **21**, 1277 (1927).
5) Kennaway, E. L., and Hiegger, I.: Biochem. J., **21**, 751 (1927).
6) Voegtlin, C., and Thompson, J. W.: J. Biol. Chem., **70**, 793, 801 (1926).
7) Fujiwara, I., Nakahara, W., and Kishi, S.: Gann, **32**, 107 (1938).
8) Iki, H.: Gann, **33**, 216 (1939).
9) Goldzieher, J. W., Pawls, W. B., and Goldzieher, M. A.: J. Biol. Chem., **203**, 519 (1948).
10) Kolthoff, I. M., and Stricks, W.: J. Am. Chem. Soc., **72**, 1952 (1950).
11) Kapalan, N.O. and Lipmann, F.: J. Biol. Chem., 174, 37 (1948).
12) Ogur, M., and Rosen G.: Arch. Biochem., **25**, 262 (1950).

要　旨

腫瘍組織の非蛋白性及び蛋白性 SH

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各種腫瘍組織について，アンペロメトリーを用いて，非蛋白性(酸溶性) SH 基，蛋白性 SH 基，さらに両者の -S-S- 基を測定した。正常肝担癌動物肝についても実験を行い比較検討したが，増殖の盛んな腫瘍組織に SH 基または -S-S- 基が多いということはなかった。また SH 基を活性基として持つコエンチーム A を酵素学的に測定し，腫瘍組織には少いことを確かめた。

(文部省科学研究費による)

[GANN, 48, 81~90; March, 1957]

ON THE BACTERIOSTATIC ACTION OF SOME BASIC PROTEIN FRACTIONS OBTAINED FROM NEOPLASTIC TISSUES

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In a previous paper we reported a method of the preparation of potent toxohormone (O-fraction), which is free from nucleic acid contamination (1). The method consists of extraction from tissue acetone powder with methanol-acetic acid mixture (9 : 6) at 75°C for 2 hours and of precipitation by the addition of the equal volume of ether to the extract. The preparation was active enough to depress liver catalase activity by injection in 20 mg dose per mouse.

Toxohormone manifests the action only *in vivo* and has inhibiting action *in vitro* on neither crude liver homogenate nor solution of crystalline catalase (2, 3). The assay of toxohormone has to be made, heretofore, by *in vivo* method using mice, which is troublesome and is attended with difficulties resulting, for instance, from individual variation of liver catalase activity.

In the course of search for a new method using microbe for the bioassay of toxohormone, we found the fact that the above described potent toxohormone concentrate (O-fraction) has a considerable bacteriostatic activity. Meanwhile, some other important evidences were obtained. It was found that TO-fraction, which was prepared by the application of the methanol-acetic acid extraction on the usual toxohormone preparation, has high catalase depressing activity in mice but has no bacteriostatic action at all. It was also found that TO-fraction does not influence either the growth rate or catalase activity of *E. coli*, whereas it has noticiable effect on the mouse liver catalase. From these facts it is concluded that bacteriostatic action and depressing activity on mouse liver catalase were caused by different substances. We may as well give up the attempt to use microbe for the bioassay of toxohormone at the present time, if there still remains the possibility that TO-fraction can not penetrate the cell membrane of *E. coli*, thus accounting for its inability to affect the catalase activity of this microbe.

In this paper, we describe the results of some experiments on the bacteriostatic action of O-fraction.

Material and Methods

The method of preparation of O-fraction was fully given in our previous paper. TO-fraction was prepared by the method also described in the same paper from

the crude toxohormone made by the method of Nakahara and Fukuoka. It was accertained that these two fractions were composed entirely of polypeptide. These were made from rhodamine sarcoma, a transplantable rat fibrosarcoma, throughout this experiment.

Escherichia coli (C 1) and *Proteus vulgaris* (HX 19) were kindly donated by Dr. Tadashi Yamamoto, Institute of Infectious Diseases, Tokyo University. They were inoculated in the medium, the composition of which was 2.5 per cent glucose, 0.5 per cent peptone, 0.1 per cent NaCl and 0.5 per cent KH_2PO_4 and was adjusted to pH 7.0. The growth of bacteria was determined nephrometrically.

Oxygen consumption was determined by the conventional method using Warburg's manometer. It should be noted that Krebs-Ringer-Phosphate solution containing no magnesium and calcium was used.

Results

Inhibition of Growth. Figure 1 shows the growth curve of *E. coli* at various concentrations of O-fraction in glucose-peptone-phosphate medium. At the concentration of 250 μg per ml, bacterial growth was completely inhibited. Figure 2 shows the growth curve of *Proteus vulgaris* (HX 19). This bacterium was not responsive to the presence of O-fraction in medium.

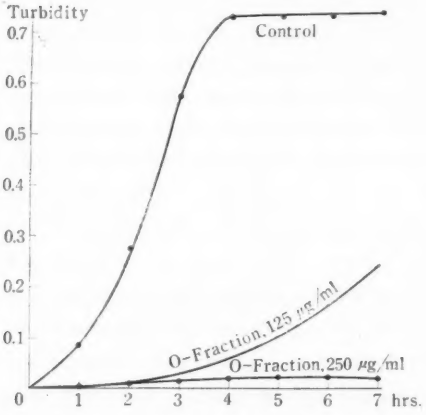


Figure 1. The effect of O-fraction on the growth of *E. coli*.

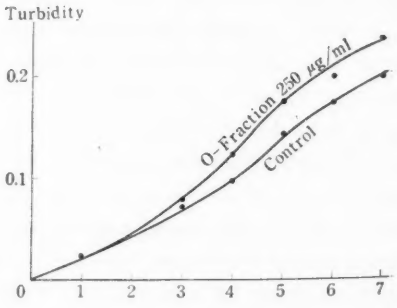


Figure 2. The effect of O-fraction on the growth of *Proteus vulgaris*.

Figure 3 represents the fact that TO-fraction has no inhibiting action on growth of *E. coli*. The catalase activity at the end of culture time with and without the O-fraction is almost the same.

In Figure 4 are illustrated the growth curves of *E. coli* at various hydrogen ion concentrations of medium with or without the addition of 250 μg of O-fraction

per ml. Almost the same degree of inhibition is recognized from pH 5.4 to 7.6.

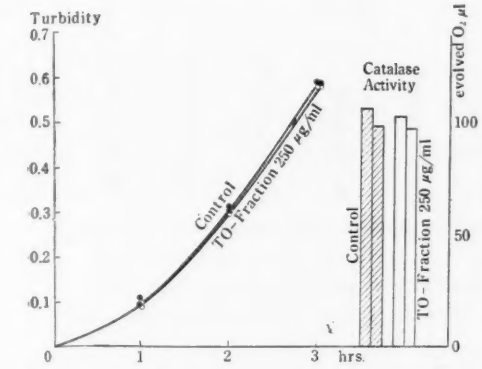


Figure 3. The effect of TO-fraction on the growth and catalase activity of E. coli.

Catalase activity was determined at the end of incubation on 0.1 ml of bacterial suspension by the method described by Beers and Sizer (Science, **117**, 710 (1950))

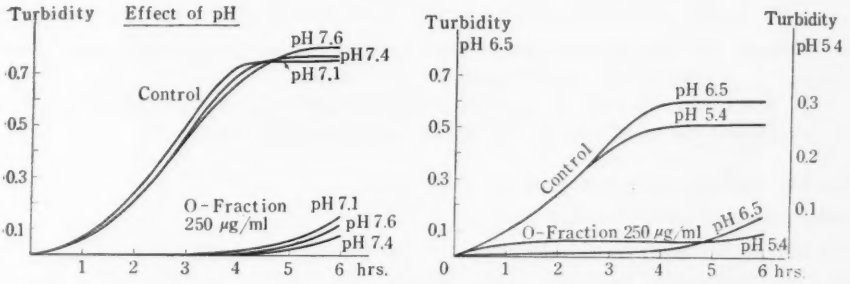


Figure 4. The effect of O-fraction on the growth of E. coli at various hydrogen ion concentrations.

Figure 5 represents the counteraction of various compounds containing sulfate ion or sulfur to the inhibiting activity of O-fraction. Dubos reported that sulfate ion has a strong counteraction to the inhibition of the growth of tuberculous bacilli produced by some basic proteins prepared from calf thymus (4). Contrary to their data, our experiments revealed that magnesium sulfate shows a protective effect against O-fraction but ammonium sulfate, potassium sulfate and sodium sulfate are without that action. Sulfur containing compounds such as cysteine, taurine and potassium rhodanate were also inert. On the other hand it was discovered that magnesium chloride had protective action against O-fraction. This was the least expected phenomenon. Next, we tried the compounds containing magnesium ion, other divalent or trivalent cations, and the results are represented in Figure 6. Magnesium sulfate, magnesium chloride, magnesium nitrate, magnesium acetate, magnesium rhodanate, calcium chloride, aluminium chloride and barium acetate all show protective action against the bacteriostatic action of

O-fraction. These compounds were added to the medium at a final concentration of 4 mM, and the concentration of O-fraction was 250 μg per ml in all the cases.

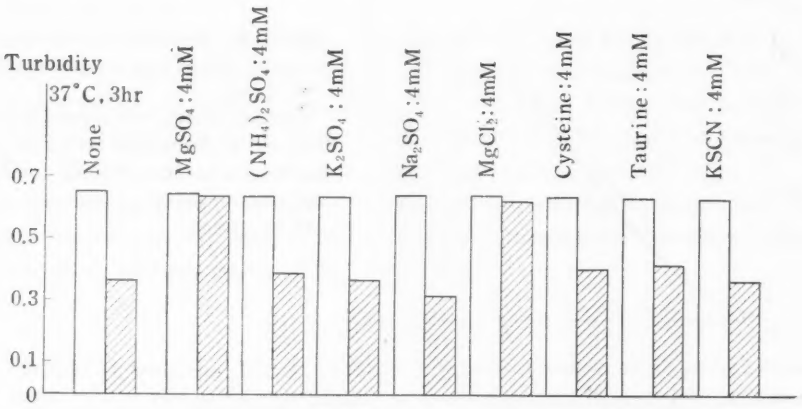


Figure 5 a. The counteraction of various compounds containing sulfate and sulfur to the inhibiting activity of O-fraction.
White bars represent the turbidity in control experiments, and shaded bars represent the tubidity in experiment with O-fraction (250 μg/ml).

Divalent cations such as magnesium, calcium and barium and trivalent cation such as aluminium have the protective action. Although Weissman reported lecithin was protective against inhibition of growth of *Bacillus anthracis* induced by calf-thymus histone, we could not recognize its protective power (5). Weissman described that bacteriocidal action of calf-thymus histone against *Bacillus anthracis* was removed through the addition of DNA but not of RNA (5). In our present experiments DNA and RNA demonstrated a similar protective action.

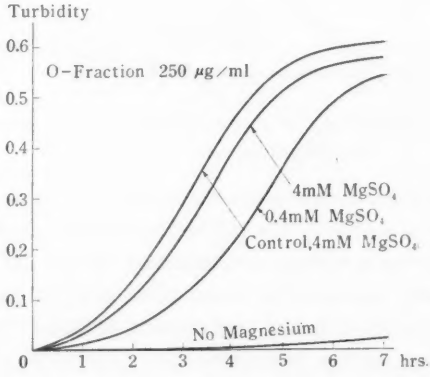


Fig 5 b. The counteraction at various concentrations of magnesium chloride to the inhibiting action of O-fraction.

Inhibition of Oxygen Consumption. In Figure 7 is illustrated the inhibition of oxygen consumption of *E. coli* by O-fraction. Sodium glutamate at the final concentration of 0.005 M and 0.001 M or glucose at the final concentration of 0.01 M was added and the concentration of O-fraction was 1.0 per cent. The inhibition

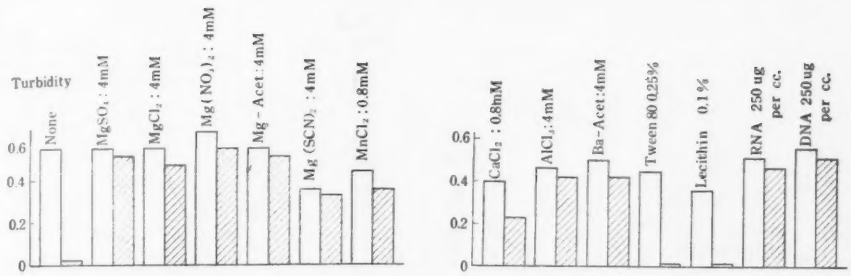


Figure 6. The counteraction of di- or trivalent cations to the inhibiting activity of O-fraction.

White bars represent the turbidity in control experiments, and shaded bars represents the turbidity in experiment with O-fraction (250 μg/ml).

of respiratory activity by O-fraction was confirmed. No difference between the degrees of inhibition at two different concentrations of glutamate was observed.

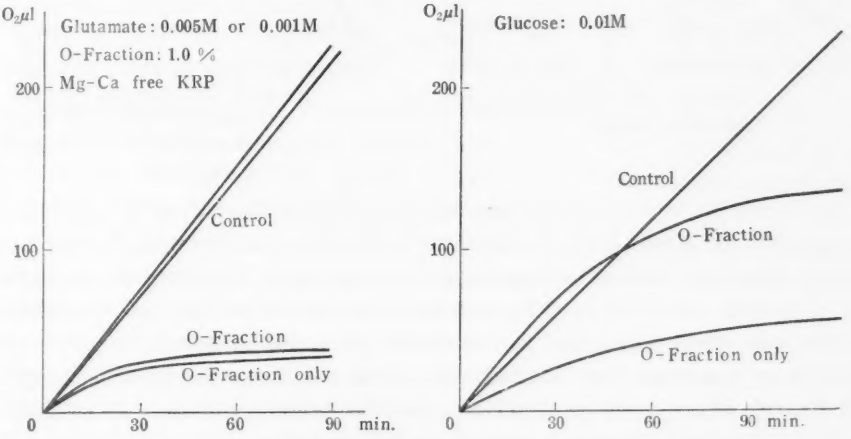


Figure 7. Inhibition of oxygen uptake of E. coli by O-fraction when sodium glutamate or glucose was added.

Figure 8 represents the oxygen consumption when alanine or aspartate were used as substrate. It is obvious that O-fraction inhibits the respiration regardless of the kind of substrate used. The delay in the onset of inhibition of oxygen consumption was commonly recognized when various substrates were used. The degree of inhibition increased with lapse of time.

Figure 9 shows the respiratory inhibition by various amounts of O-fraction in the presence of glutamate as substrate. The higher the concentration of O-fraction, the less was the delay in the onset of inhibition.

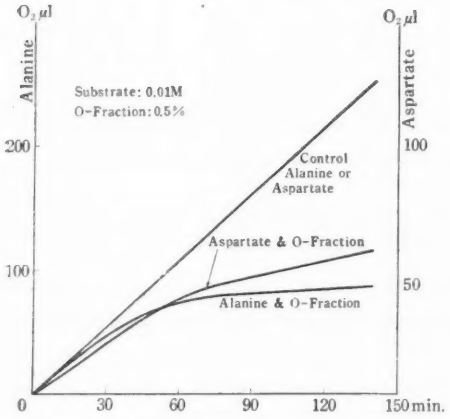


Figure 8. Inhibition of oxygen uptake by O-fraction when alanine or aspartate was added.

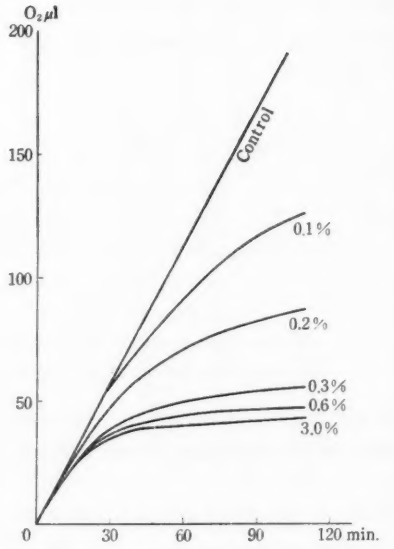


Figure 9. Inhibition of oxygen uptake of E. coli at various concentrations of O-fraction when sodium glutamate (final 0.005 M) was added.

In Figure 10 is shown the removal of respiratory inhibition by addition of magnesium ion tipped from the side-arm of vessels at different times in the course of experiments. 20 mM of magnesium sulfate completely eliminated the inhibition of respiration produced by O-fraction and increased respiratory rate to the level of the case where magnesium sulfate alone was added without O-fraction. The tipping at 50 minutes after the beginning of the run of experiment was completely ineffective. The tipping at 20 minutes after the beginning of the run of experiments still proved effective in eliminating the inhibition.

Next, we carried out the experiments, in which three different mixtures of glutamate plus bacteria, bacteria plus O-fraction, and O-fraction plus glutamate were first incubated for fourty minutes, after which time, O-fraction, glutamate and bacteria respectively were added from the side arm. We could not find any difference among these three mixtures after the addition of the last component. The results of these experiments are illustrated in Figure 11.

We also carried out an experiment on the elimination by RNA of the inhibition of oxygen consumption by O-fraction in the presence of glutamate as substrate. Strangely enough, it was found in this experiment that the oxygen consumption was much higher when O-fraction plus RNA were added than when RNA or O-

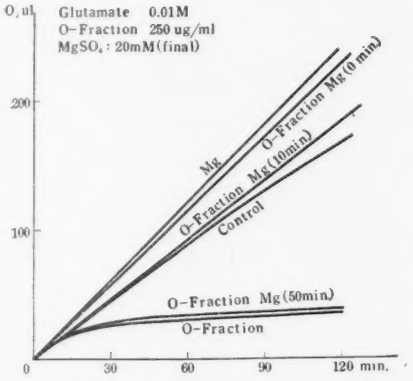
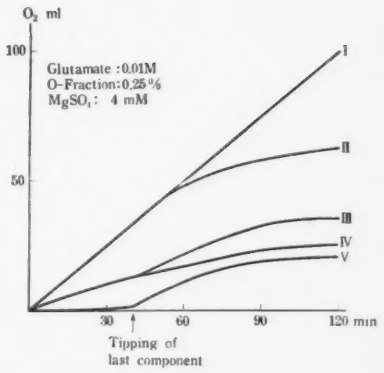


Figure 10. The removal of respirtoray inhibiting action of O-fraction by magnesium ion.

Figures in parenthesis represent the time when magnesium was tipped from side arm.



components in preincubation	component tipped at the time marked with arrow
I glutamate, bacteria	none
II glutamate, bacteria	O-fraction
III O-fraction, bacteria	glutamate
IV O-fraction, bacteria	none
V O-fraction, glutamate	bacteria

Figure 11. Experiments of Preincubation.

fraction only was added. It is possible that O-fraction contains some substrate increasing oxygen consumption together with the bacteriostatic substance, and the former was utilized by bacteria as the results of the removal of O-fraction action by RNA. The results are illustrated in Fig. 12.

Figure 13 represents the effect of O-fraction on the respiration of *Proteus vulgaris* in the presence of glutamate or glucose as substrate. O-fraction did not inhibit the oxygen uptake but rather enhanced it.

Adsorption of O-Fraction by the Microbes. It was noted in the first part of our experiments that while the growth of *E. coli* is definitely inhibited by O-fraction, that of *Proteus vulgaris* is not at all affected. In order to throw light, if possible, on the mechanism of this discrepancy, we next examined the adsorption of O-fraction on the surface of cells of *E. coli* and of *Proteus vulgaris*, inhibited and not inhibited respectively by O-fraction. 18.75 mg of O-fraction was dissolved in 10 ml of 0.01 M phosphate buffer pH 7.0 and *E. coli* and *P. vulgaris*, in the amounts of 38.5 and 41.2 mg dry weight respectively, were added and left to stand for one hour at room temperature, after which they were centrifuged at 6000 rpm for 10 minutes and the amount of protein in the supernatant was determined by biuret reaction. The amount of protein was 14.00 and 16.55 mg respectively. It was clear that certain amounts of O-fraction, namely, 4.74 mg in the case of *E. coli* and 2.35 mg in the case of *Proteus vulgaris*, were precipitated with bacterial cells. It is worthy of attention that more polypeptide was adsorbed by

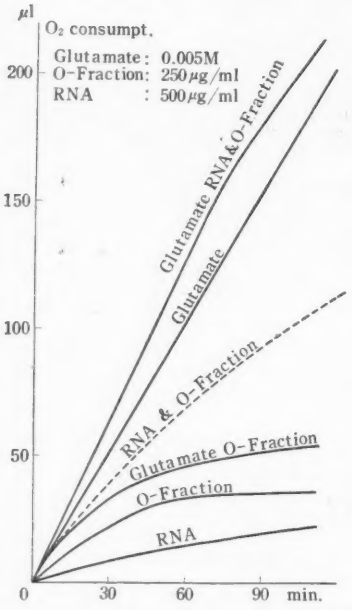


Figure 12. The removal of respiratory inhibiting action of O-fraction by RNA.

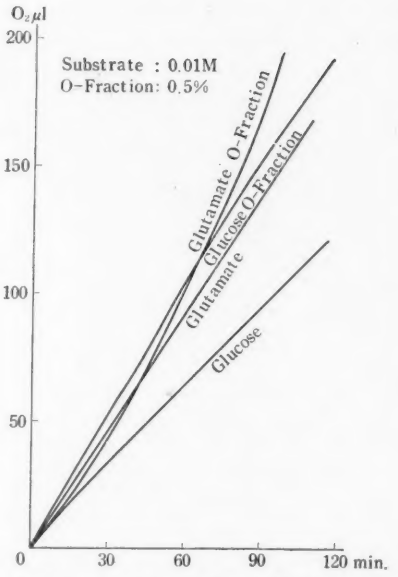


Figure 13. The effect of O-fraction on oxygen uptake of Proteus vulgaris.

E. coli, the growth of which is inhibited, than by ***Proteus vulgaris***, which is not inhibited by the O-fraction component.

Discussion

In the experimental results described above, the bacteriostatic action was proved of O-fraction, which was obtained by extraction from tumor tissue acetone powder with methanol-acetic acid mixture for 2 hours at 75°C and precipitation after the addition of the equal volume of ether to the extract.

The substance in O-fraction which contributes to the anti-bacterial action is not yet known. Further purification of O-fraction will clarify this point. Based on the fact that such basic protein as histone and protamine have an anti-bacterial action (4-7), we suspect that the anti-bacterial action of O-fraction may be caused by histone-like substance of tumor tissue. Further research comparing the anti-bacterial activity of such basic protein as histone of tumor tissue with one of normal tissue would be necessary. There have been many auguments as to whether or not histone of the tumor tissue differs from that of normal one, but no definite conclusion has yet been obtained. Further studies in this laboratory may contribute to the solution of this problem.

TO-fraction does not inhibit either the growth or synthesis of catalase of *E. coli*, whereas it has a remarkable catalase depressing action in mouse liver. We can thus conclude that anti-bacterial action and mouse liver catalase depressing action, namely toxohormone action, are due to different substances. Another recent experimental result that by pepsin digestion the O-fraction lost completely the anti-bacterial action without losing the toxohormone activity at all, supports the above conclusion. Although the mechanism of this bacteriostatic action cannot be fully explained, there is a possibility that the active substances are adsorbed on the surface of bacterial cells, thus inhibiting the utilization of substrate. This assumption is supported by the following experimental results: (1) Oxygen consumption of *E. coli* was inhibited by O-fraction, regardless of the kind of added substrate. (2) The complete inhibition of oxygen uptake occurred with a considerable time lag after the start of experiment. It is presumed that the adsorption has been completed by this time. (3) While magnesium ion added at the biginning of the run of experiments removed the inhibition of oxygen consumtion by O-fraction, magnesium ion added after the 50 minutes preincubation of bacterial cells and O-fraction had no such effect. (4) More O-fractions was precipitated with *E. coli* than with *Proteus vulgaris*.

The mode of protective action of divalent and trivalent cations against O-fraction would be explained by the competetion with bacterial cells. Neutralization of basic protein by RNA and DNA accounts for their protective action against O-fraction.

Summary

1. O-fraction obtained from tumor tissue inhibited the growth of *E. coli* but not of *Proteus vulgaris*.

2. TO-fraction obtained from tumor tissue did not inhibited either the growth or the synthesis of catalase of *E. coli*.

3. Various divalent or trivalent cations and RNA or DNA removed the growth inhibiting action of O-fraction.

4. Oxygen consumption of *E. coli* was inhibited by O-fraction regardless of the kind of added substrate.

5. The inhibition of oxygen consumption was removed by magnesium ion and RNA or DNA.

6. The nature of anti-bacterial principle and its mode of action were discussed.

The authors wish to acknowledge the encouragement of Dr. W. Nakahara, Director of Cancer Institute, during the course of this work.

References

1. Ono, T., Sugimura, T., and Umeda, M.: Gann, **46**, 617 (1955).
2. Nakahara, W., and Fukuoka, F.: Gann, **40**, 45 (1949).
3. Endo, H., Sugimura, T., Ono, T., and Konno, K.: Gann, **46**, 51 (1955).
4. Hirsch, J. G., and Dubos, R. J.: J. Exptl. Med., **99**, 65 (1954).
5. Weissman, N., and Graf, L.: J. Infect. Disease: **80**, 145 (1947).
6. Miller, B. F., Abrams, R., Dorfman, A., and Klein, M.: Science, **93**, 428 (1942).
7. Felix, K., and Harteneck, A.: Z. f. physiol. Chem., **157**, 76 (1926).

要　旨

腫瘍組織から得たある塩基性蛋白の抗菌性について

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(癌研究所)

トキソホルモンのバイオアッセーに，細菌を使う試みを研究している途次に，腫瘍組織から得たO分劃（アセトン粉末より，メタノール醋酸 75°C 2時間抽出，エーテルで沈澱させる）はかなり強い抗菌力のあることを見出した。一方トキソホルモンにメタノール，醋酸抽出法を応用して得たTO分劃は，トキソホルモン作用は強いが抗菌性はみとめられなかったから，両作用は別種の物質でおこる。

O分劃は E. coli の生長を阻害するが，Proteus vulgaris の生長を阻害しない。またO分劃はグルコース，グルタメート，アラニン，アスパルテートを基質とした場合その酸素消費を抑制する。Proteus vulgaris の場合にはこのような事実も見出せない。O分劃による E. coli の生長阻害及び酸素消費抑制はマグネシウム，カルシウム，アルミニウム等のイオン，RNA, DNA によって打消される。

O分劃は E. coli の菌体に吸着し抗菌性を表わすものと考える。

（文部省科学研究費による）

[GANN, 48, 91~100; March, 1957]

PURIFICATION OF TOXOHORMONE

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In a previous report of the authors published in this journal (1), it was demonstrated that potent toxohormone preparations free from nucleic acid can be obtained from the acetone dried powder of several malignant tumor tissues. It was also established that the same procedure was applicable to the raw toxohormone preparation, obtained by the original procedure of Nakahara and Fukuoka (2), for obtaining a satisfactory preparation, which was designated as TO-fraction by the authors. TO-fraction was also free from nucleic acid, and was composed almost entirely of polypeptide.

For the purpose of further purification, alcohol fractionation, zone electrophoresis and ion exchange resin column chromatography were tried, but they failed to give a complete separation of impurities without substantial losses of the active material. However, the cellulose powder column adsorption technique applied to the TO-fraction obtained from rhodamine sarcoma yielded a fraction effective in 1 mg doses in depressing the liver catalase activity of the mouse *in vivo*. From the corresponding fraction of Murphy lymphosarcoma the same technique yielded a fraction effective in doses so small as 0.5 mg.

With the hope of elevating the percentage of recovery of the active material in the cellulose adsorption technique, TO-fraction was subjected to pepsin digestion and subsequently precipitated with picric acid in a weakly alkaline medium preliminary to the process of adsorption. Unexpectedly, the precipitate formed on the addition of picrate took the shape of fine needle crystals, which were revealed to be active in 0.1–0.2 mg doses for mice.

In this paper the details of the procedures of cellulose powder column adsorption and of the picrate formation will be described, and the amino acid composition of each sample will be presented.

EXPERIMENTAL

A) Purification by Adsorption on Cellulose.

Cellulose powder of high quality made for chromatographic use by W. & R. Balston Company, London, was washed serially with 0.1 N HCl, H_2O, and 0.1 N acetic acid. Several combinations of column sizes and quantities of starting TO-fraction were tested as demonstrated in Table 1. In an illustrative experiment

Table 1. Cellulose Powder Adsorption of TO-Fraction.

Exp. No.	Source of material	Dimension of column	Amount of TO-Fraction	Amount recovered		
				in acetic acid	in 0.1 NHCl	in 0.2 NHCl
I	Rhodamine sarcoma	1.5×35cm	250mg	220mg (88%)	12.3mg (5.6%)	—
II	〃	1.5×35	750	548 (73)	13.0 (1.8)	—
III	〃	2.5×35	250	209 (83.5)	11.2 (4.5)	—
* IV	Murphy lymphosarcoma	2.5×35	160	150 (94)	7.4 (4.6)	—
** V	Rhodamine sarcoma	2.4×35	450	—	184 (41)	40.3mg (8.9%)

* In Exp. IV, methanol-acetic acid extract of Murphy Lymphosarcoma was tested with preliminary digestion by pepsin.

** Oxycellulose powder column was employed in this experiment.

(Exp. I in Table 1) a chromatographic column 1.5 cm in diameter and 35 cm high was prepared with purified powdered cellulose, which was poured as thick slurry in 0.1 N acetic acid. 250 mg of TO-fraction, prepared from rhodamine sarcoma, was dissolved in 10 ml of 0.1 N acetic acid and after application to the column was washed through with 100 ml of 0.1 N acetic acid. The eluate was collected by the automatic fraction collector in 5 ml fraction in each tube. 220 mg of the original material (as estimated by the biuret reaction) emerged with the acetic acid. 100 ml of 0.1 N HCl was then run through, which eluated 12.3 mg of polypeptide as estimated by the biuret reaction. The fractions containing significant concentration of material were combined and dried while frozen, acetic acid washings and HCl eluate separately. We refer to the former as Peak I and the latter as Peak II (Fig. 1).

The activity of each peak was assayed as described in the previous report (1). As shown in Fig. 2, Peak I exhibited only ambiguous activity in 10 mg doses, but on the other hand, Peak II

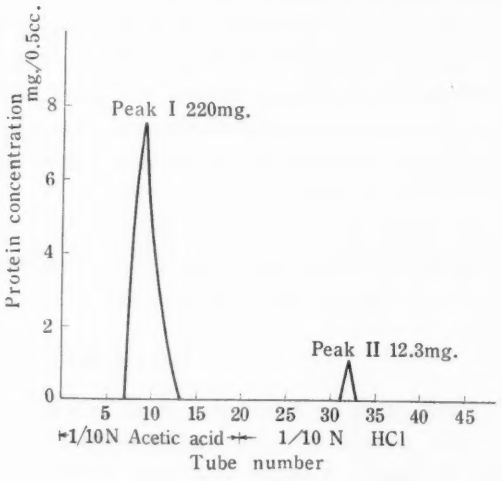

Fig. 1. Cellulose Column Chromatography (Exp. I) Rhodamine sarcoma TO-fraction 250mg. Pile 1.5×35cm.

in every experiment was distinctly effective in 1 mg doses but not so effective in 0.5 mg doses, indicating a potency of about 10 times that of the starting TO-fraction, which was effective in 10 mg doses as demonstrated in Fig. 2.

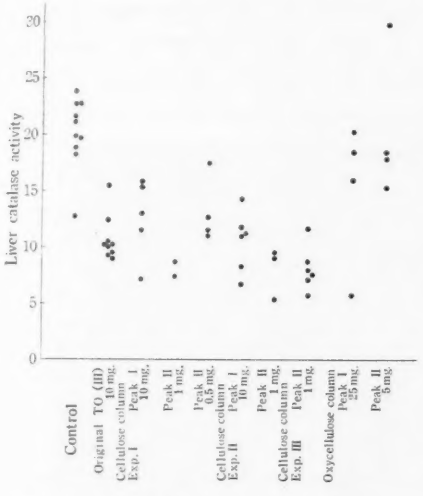

Fig. 2. The effect of various cellulose column chromatographic fractions on liver catalase activity in mice. (Rhodamine sarcoma)

Among the three experiments listed in Table 1, the procedure of Exp. I may be considered to be most excellent in the recovery of potency, that is Exp. II with more starting material in same scale of column or Exp. III with same quantity of starting material in larger scale of column yielded approximately equal amount of Peak II of the same potency, that is, effective in 1 mg doses.

In Exp. IV of Table 1, methanol-acetic acid extract of Murphy lymphosarcoma was also subjected to this cellulose adsorption technique. 160 mg of the extract was digested by pepsin prior to the application on column. As represented in Fig. 3, it was devided by this procedure into two fractions, Peak I and II, as in the case of rhodamine sarcoma, and Peak II was revealed to be active in so small a dose as 0.5 mg per mouse. A comparative assay of Peak II with the original preparation of Murphy lymphosarcoma is presented in Fig. 4.

Exp. V in Table 1 was carried out by means of oxycellulose column in the place of cellulose powder column employed in the above experiments. Oxycellulose was prepared by the oxidation of cellulose powder with sulfric acid-permanganate mixture according to the procedure of Knecht and Thompson (3). The adsorption power of oxycellulose was so strong that 0.1 N acetic acid washing could not bring out any portion of the TO-fraction applied on the column, and so 0.1 N HCl and 0.2 N HCl were run successively through it. Only small portions of the starting material were recovered by this procedure, and they were revealed to be of no activity, as demonstrated in Fig. 2.

B) **Preparation of Picrate Crystals.**

It has been firmly established by Nakahara and Fukuoka that pepsin digestion does not destroy the potency of toxohormone, but only converts it into dialysable smaller fregmants (4). It was considered to be more convenient to handle the polypeptide hormone in its relatively low molecular form if possible, as demon-

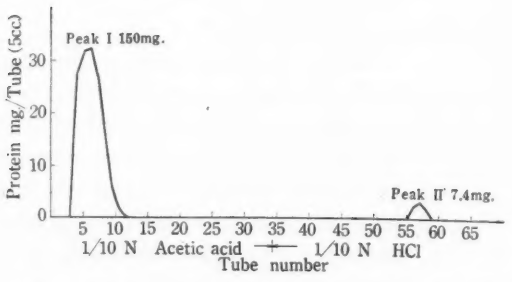

Fig. 3. Cellulose column chromatography (Exp. V) of Murphy lymphosarcoma O-fraction (160 mg.) (pepsin digested beforehand) Pile 2.5×35cm.

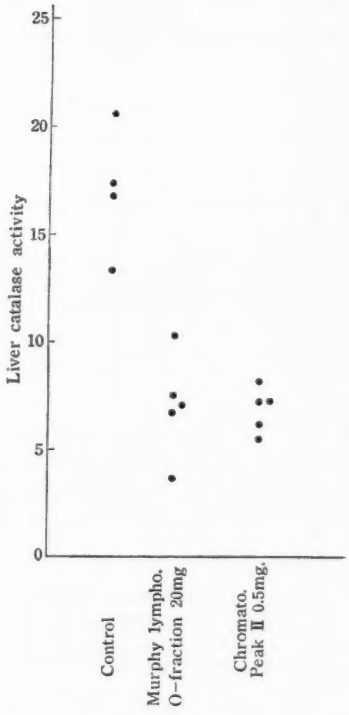

Fig. 4. Effect on liver catalase activity in mice of O-fraction and its cellulose column chromatographic Peak II prepared from Murphy lymphosarcoma.

strated in the purification of adrenocorticotropic hormone (5, 6). In the case of Murphy lymphosarcoma as described in the preceding section, by introducing pepsin digestion, 20 to 40 times purification was achieved practically by one step of cellulose adsorption. In the attempt to fractionate further the pepsin digest before submitting it to chromatography, it was recalled that Felix *et al.* divided the pepsin digest of histone into several fractions by classical precipitation technique, and that they obtained a basic fragment designated as histopeptone by means of picrate precipitation from a weakly alkaline medium (7).

We, therefore, adopted their procedure in this experiment as follows: TO-fraction obtained from rhodamine sarcoma was incubated with pepsin (soluble powder, Merck Co.), 1 per cent of its weight, in 0.1 N HCl for four days at 37°C under toluol. At the end of the incubation it was boiled for 5 minutes and centrifuged to remove the small amount of precipitate. Then the reaction of the clear supernatant was adjusted to pH 8.3 (with the aid of phenolphthaleine and thymole blue indicator paper), and as much as 1/3 of its volume of saturated Na-picrate solution was added. In the course of 30 minutes after the addition of

picrate, precipitate with sheen appeared gradually. By microscopic examination it was revealed to be fine needle crystals, as illustrated in Fig. 5. Starting from 1 g of TO-fraction, the yield of picrate was about 10 mg. Three preparations were obtained by this procedure, and all of them consisted of crystals similar to those shown in Fig. 5. The activities of these crystalline picrates were assayed after removal of picric acid moieties, and they were revealed to be active in 0.1–0.2 mg doses, i. e., about 50 times as active as the original TO-fraction. Fig. 6 represents the data which indicate the effect of these three similar samples of crystalline picrates.

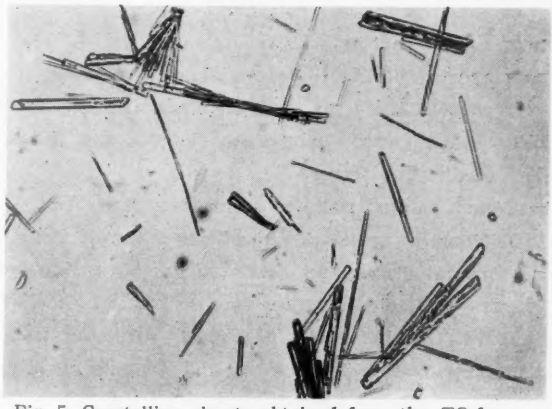

Fig. 5. Crystalline picrate obtained from the TO-fraction of rhodamine sarcoma.

The same procedure was also applied to the methanol-acetic acid extract, instead of TO-fraction, of rhodamine sarcoma and it yielded a picrate precipitate, which contained some crystals resembling those shown in Fig. 5 but the material was contaminated so heavily with amorphous picrates that it was discarded without biological test.

Some chemical properties of the crystalline picrate were investigated. It is sparingly soluble in water especially in cold, but soluble in dilute mineral acids and more soluble in aqueous alcohol, e. g., water saturated 2-butanol. On heating it decomposes at over 300°C, showing no melting point. Its nitrogen content is estimated to be about 13% by the Kjeldahl nesslerisation

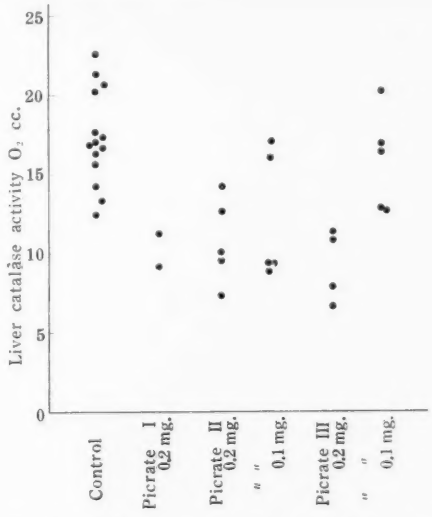

Fig. 6. Effect of three samples of crystalline picrate on liver catalase activity

method. It gives very weak and delayed ninhydrin reaction, but after being hydrolysed by 1 : 1 HCl, it turns to react intensively and promptly with ninhydrin.

In the paper chromatographic test, this picrate gives a single spot which is developed by the spray of ninhydrin solution. Its Rf value is 0.20 in butanol: acetic acid: water solvent and 0.016 in lutidine: water system and in either case it does not coincide with that of any amino acid.

The amino acid composition of the picrate is presented in the following section, where a comparison is made with those of other fractions.

C) **Amino Acid Composition of Each Fraction.**

Each fraction (2 to 5 mg) was hydrolyzed at 110°C in a sealed evacuated tube with 1 : 1 HCl which has been glass-distilled to remove trace metals. After hydrolysis for 24 hours, the acid was evaporated over a steam bath, and the dry residue was taken up with 3.0 ml of distilled water.

The amino acid composition of each sample was determined by converting the hydrolysate to N-dinitrophenyl (DNP)-amino acids and submitting these derivatives to quantitative two-dimensional chromatography on filter paper (8), which was carried out as follows: the papers applied with ether soluble fraction of DNP-amino acids were run overnight by the ascending procedure with the toluol-chloroethanol-pyridine-0.8 N ammonia system of Biserte and Osteux (9) in the first dimension, then run overnight in the second dimension by the descending procedure with 1.5M phosphate buffer at pH 6. The spots with a set of appropriate blank were cut out and dropped into test-tubes. 4 ml of distilled water was pipetted into each of the tubes, which were then placed in a water bath at 55 to 60°C for 15 minutes to allow the elution of the color. After an additional 15 minutes for the solutions to cool to room temperature, they were centrifuged, and the optical density of each supernatant was read in the Beckman model DU spectrophotometer against a water blank at 360 mμ in the case of most DNP-amino acids and at 385 mμ in the cases of DNP-proline and DNP-hydroxyproline. The optical densities obtained were converted to molar ratios, by multiplying by the set of factors F employed by Levy (8).

Although tryptophan is destroyed by acid hydrolysis, it has been already confirmed by the previous examination of the ultraviolet light absorption that methanol-acetic acid extract of acetone powder and TO-fraction were devoid of tryptophan as well as tyrosine (1), so no endeavour was made to evaluate tryptophan contents of these fractions. Since DNP-arginine cannot be extracted by ether in the above procedure, the arginine content of the hydrolysate was determined independently by Sakaguchi's reaction.

The reliability of this procedure was ascertained by the fact that the amino acid composition of casein (nach Hammarsten, Merck Co.) determined prelimi-

narily by the same procedure was confirmed to coincide exactly with that established by previous workers (10).

The amino acid compositions of methanol-acetic acid extract of acetone dry powder (O-fraction), TO-fraction, 0.1 N HCl eluate from cellulose column (TOC-fraction) and crystalline picrate, which were all prepared from rhodamine sarcoma, were determined by the paper-DNP technique described above and their number of amino acids residues per 1 mol. of threonine were represented in Table 2.

Because O- and TO-fraction are still very crude, it must be of little worth to ponder on their amino acids composition, but it is of interest that they exhibit

Table 2. Amino acid compositions of O-, TO, TOC-fractions, Crystalline Picrate and Collagen.

	O-fraction	TO-fraction	TOC-fraction*	Picrate**	Collagen
Hydroxyproline	1.21	5.1	0.65	(2) 2.10	5.3
Proline	2.45	6.8	1.52	(2) 2.30	6.6
Threonine	1	1	1	(1) 1.20	1
Valine	1.13	1.03	1.16	(1) 1	1.45
Leucine and Isoleucine	2.45	2.18	3.78	(2) 1.83	2.10
Phenylalanine	0.675	0.62	0.67	(2) 1.90	1.25
Alanine	3.56	5.35	4.2	(4) 4.30	5.30
Glycine	5.7	18.5	12.9	(5) 5.10	17.5
Serine	0.891	2.63	1.77	(2) 1.97	1.7
Lysine	2.21	2.35	2.7	(2) 1.99	1.6
Glutamic acid	2.45	3.42	6.86	(5) 5.26	3.65
Aspartic acid	2.56	6.19	4.52	(2) 2.12	2.3
Cystine	0.115	0.056	0.00	(0) 0.00	0.00
Methionine	0.00	0.00	0.00	(0) 0.00	0.25
Tyrosine	0.37	0.00	0.00	(0) 0.00	0.40
Tryptophane	0.00	0.00	0.00	(0) 0.00	0.00
Arginine	—	4.30	—	(2) 1.87	2.73

* HCl eluate from cellulose powder column.

** The figures in the parentheses represent the residues to nearest integer.

particular characteristics in containing very small amounts of cystine, methionine, tryptophan and tyrosine. Besides these features, the existence of hydroxyproline in them strongly suggests the contamination of collagen. The content of this amino acid in TO-fraction is as high as in collagen, and, moreover, a very close similarity in other amino acids composition is also observed between them, as demonstrated in Table 2.

Needless to add that collagen or gelatin obtained in the market does not exhibit any catalase depressing action even in so high a dose as 50 mg.

The hydroxyproline content of the HCl eluate fraction (TOC in Table 2) is

reduced considerably from that of TO-fraction, but it is also contained in the picrate. The presence of hydroxyproline in picrate will be referred to again in the following section.

Regarding the amino acid composition of the picrate, it is at once evident that most of the amino acid residues are in the stoichiometric ratios and that the picrate contains a minimum of thirty-two amino acid residues. The picrate is lacking in cystine, methionine, tryptophan and tyrosine.

Discussion

It was revealed in this laboratory that the TO-fractions prepared from the ascites tumor cells of Yoshida sarcoma and hepatoma, besides the solid tumor such as rhodamine sarcoma, also contain hydroxyproline as their constituent, but that the similar fraction obtained from Ehrlich ascites tumor cells does not contain it. Recently the authors have reported the amino acid composition of a toxohormone fraction propared from the urine of patients suffering from gastric cancer. No hydroxyproline was detected in that fraction (11). Although some German workers (14) reported recently the presence of hydroxyproline in the protein of a bacteriophage, only collagen and gelatin, among animal substances, have been known to contain this amino acid in their molecules and so it may be safe to conclude that the hydroxyproline contents of the above fractions are derived from the impurities in them.

The presence of hydroxyproline in the picrate is one of the objections to its homogeneity. The peptide in the picrate takes the shape of fine crystals, shows a single spot in paper chromatography and contains amino acid residues nearly in stoichiometric ratios, as described in the experimental section, but other convincing evidences are lacking as yet to prove its homogeneity. There have been known some crystalline polypeptides and/or proteins revealed to be not homogenous by counter-current distribution test and partition chromatography (12).

Kosuge *et al.* (13) have already reported a fraction named as KNA, which was prepared from the raw toxohormone preparation as ribonucleic acid fraction containing a little protein, and which was active in as small a dose as the picrate reported here. There are some reasons to believe that the protein moiety of their fraction might be responsible for the toxohormone action. If that is the case, toxohormone in its pure form may be a more active peptide than that of our crystalline picrate.

Fortunately the picrate obtained in this experiment is highly soluble in 2-butanol-water and is considered to afford opportunities for purification by counter-current distribution technique. The work along this line is now in progress in this laboratory.

Summary

Starting from TO-fraction, a nucleic acid-free polypeptide fraction obtained by methanol-acetic acid extraction of a crude toxohormone preparation, the active substance was concentrated by means of adsorption on powdered cellulose from 0.1 N acetic acid and elution with 0.1 N hydrochloric acid. The hydrochloric acid eluted fraction (Peak II) obtained from TO-fraction of rhodamine sarcoma was active in 1 mg doses, indicating 10-fold increase in potency. The same fraction obtained from methanol-acetic acid extract of Murphy lymphosarcoma, which was digested with pepsin prior to the adsorption on cellulose, was active in 0.5mg doses.

Crystalline picrate of an active peptide was prepared from TO-fraction of rhodamine sarcoma by the procedure of pepsin digestion and subsequent precipitation with picric acid in a weakly alkaline medium. This crystalline picrate was revealed to be active in 0.1-0.2 mg doses for mice.

The amino acid composition of each fraction was determined by paper-DNP technique.

The homogeneity of the crystalline picrate was discussed and the possibility of obtaining more active peptide than that presented in this paper was suggested.

Our hearty thanks are due to Dr. Waro Nakahara for his kind interest and encouragement.

References

1. Ono, T., Sugimura, T. and Umeda, M.: Gann, **46**, 617 (1955)
2. Nakahara, W. and Fukuoka, F.: Gann, **40**, 45 (1949)
3. Krecht, E., and Thompson, L.: J. Soc. Dyers and Col., **36**, 251 (1920)
4. Nakahara, W., and Fukuoka, F.: Gann, **41**, 47 (1950)
5. Payne, R. W., Raben, M. S., and Astwood, E. B.: J. Biol. Chem., **187**, 719 (1950)
6. Bazemore, A. W., Richter, J. W., Ayer, D. E., Finnerty, J., Brink, N. G., and Folker, K.: J. Am. Chem. Soc., **75**, 1949 (1953)
7. Felix, K.: Z. phy. Chem.: **120**, 94 (1922)
8. Levy, A. L.: Nature, **174**, 126 (1954)
9. Biserte, G., and Osteux, R.: Bull. soc. Chem. biol., **33**, 50 (1951)
10. Gordon. W. G., Semmett, W. F., and Morris, F.: J. Am. Chem. Soc., **71**, 3293 (1949)
11. Fuchigami, A., Umeda, M., Ono, T.: Gann, **47**, 295 (1956)
12. Gregory, J. D., and Craig, L.: J. Biol. Chem., **172**, 839 (1948); Tallan, H. H., and Stein, W. H.: J. Biol. Chem., **200**, 507 (1953)
13. Kosuge, T., Tokunaka, H., and Nakagawa, S.: Hokkaido J. Med. Soc.: **29**, 185 (1954)
14. Koch, G., and Weidel, W.: Z. phys. Chem. **303**, 213 (1955)

要　旨

トキソホルモンの精製

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前報において，粗製のトキソホルモンをメタノール：醋酸で抽出することにより，核酸を含まず大部分 polypeptide よりなる分劃が得られ，しかも元の活性がほぼ完全にこの分劃（TO-分劃）に回収されることを報告した。その後これをさらに次の操作により精製した。

すなわち，ローダミン肉腫より得た TO-分劃を精製粉末セルローズ柱に吸着させ，1/10 N 塩酸で溶出することにより 1mg でカタラーゼ活性を生体内で低下せしめる分劃が得られた。また Murphy の lymphosarcoma のアセトン乾燥材料をメタノール：醋酸で抽出し，あらかじめペプシンで消化してから，上のセルローズ吸着をおこない，0.5mg でも有効な分劃を得た。次にローダミン肉腫よりの TO-分劃をペプシンで消化し pH を弱アルカリ性としてから，1/3 容の飽和ピクリン酸溶液を加えたところ，0.1～0.2mg で有効な peptide のピクリン酸塩が結晶として得られた。

この結晶ピクラート中の peptide はチロヂン，トリプトファン，メチオニン，チスチンを除く各アミノ酸をふくみ，その最小単位は 32 のアミノ酸残基からなることが分った。またペーパークロマトで単一の Spot を示したが，さらに向流分配分析，partition chromato 等によりその均一性の検定と，より一層純化することを考えている。（文部省科学研究費による）

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METABOLIC PATTERN OF SILICOTIC NODULES PRODUCED IN EXPERIMENTAL ANIMALS

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It is well known that fairly large nodules are formed on the omentum if free silica powder is introduced into the peritoneal cavities of rats or guinea pigs (1). The type of reaction cells against silica powder is different according to the species of experimental animals (2). The nodule in the rat is composed of histiocytes, fibroblasts, fibrocytes and argyrophilic fibers in the early stage of its development, but in the late stage, these cell components are seen only in the peripheral zone of the nodule and the central area is replaced by the well developed collagen strands. On the other hand, the nodule in the guinea pig is a granuloma composed of epithelioid cells and multinucleated giant cells, and fibroblasts or fibers are seen only in the peripheral zone of the nodule in the very early stage.

We have studied some aspects of the metabolic pattern of these two types of silicotic nodules in order to clarify the pathogenicity of silica and to compare them with malignant tumors.

Material and Methods

1) **Silicotic Nodules of Rats.** Free silica used for rats was natural quartz of 99.7 % purity, ground to the size range under five microns. 100 mg of this quartz powder was suspended in 1 cc of saline, and injected into the peritoneal cavity of each rat. Animals were sacrificed by decapitation 6 months after the inoculation of the powder. The silicotic nodules separated from the omentum were stripped off the surrounding tissues, and the halves of these nodules were sliced for the assay of glycolysis and homogenized for succinoxidase assay, and the other halves were examined histologically.

2) **Nodules of Guinea Pigs.** Silica obtained by the hydrolysis of ethylorthosilicate was calcined for 2 hours at 1000°C. The calcined free silica was powdered to the size under 3 microns, and injected into the peritoneal cavities of guinea pigs in the same amount as in the case of rats. Animals were sacrificed 2 months after the inoculation of dusts, and nodules produced on the omentum were separated

and treated as in the former case.

3) **Assay of the Enzymatic Activities.**

a) Anaerobic glycolysis: Anaerobic glycolysis was assayed manometrically by the procedure of Warburg (3). 100 mg in wet weight of slices was subjected to the assay of anaerobic glycolysis. The activity was presented by the amount of CO_2 μl per 1 mg N of slices used for assay.

b) Succinoxidase activity: Succinoxidase activity was assayed manometrically by the technic as described by Schneider and Potter (4), and 0.2 cc of 5 % tissue homogenate in water was used per vessel. The activities were compared by the amount of μl of O_2 uptake per mg N of the homogenate.

Results

1) Anaerobic Glycolysis. Anaerobic glycolysis activity of rat's silicotic nodule was compared with those of muscle, liver, and rhodamine sarcoma of rats. Activities of these tissues are shown in Fig. 1. Among these tissues, rhodamine sarcoma exhibited the extraordinary high activity, but the activities of muscle and liver were scarecely detected in this system. Rat's silicotic nodule showed a moderate glycolytic power, which was estimated to be about one-half of that

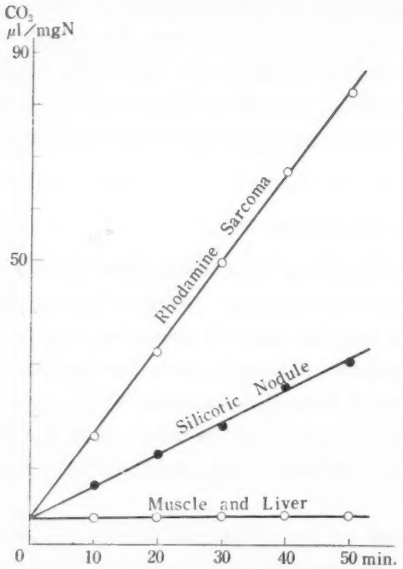

Fig. 1. Anaerobic Glycolysis of the Slice of Silicotic Nodule Produced on the Omentum of Rat.
(Otto Warburg system)

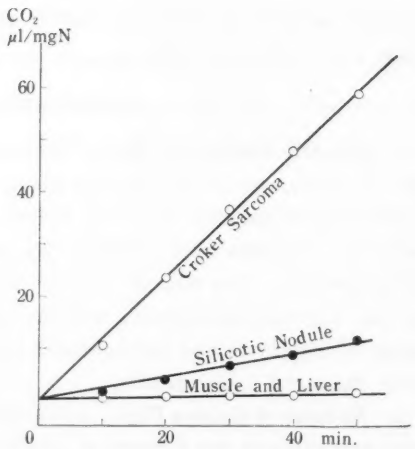

Fig. 2. Anaerobic Glycolysis of the Slice of Silicotic Granuloma Produced on the Omentum of Guinea-pig.
(Otto Warburg system)

of rhodamine sarcoma. Anaerobic glycosis activity of guinea pig's nodules, compared with those of muscle and liver of guinea pig's and Croker sarcoma of the mouse, is shown in Fig. 2, which shows that Croker sarcoma has a high activity of anaerobic glycosis, and muscle and liver so low as to be almost negligible. Activity of silicotic nodule was far feebler than that of Croker sarcoma, but was quite distinct when compared with the almost undetectable activities of muscle and liver.

2) **Succinoxidase Activity.** Succinoxidase activities of silicotic nodule, liver and spleen of rats are shown in Fig. 3. Liver has the highest activity and silicotic nodule the lowest. Fig. 4 shows the succinoxidase activities of liver, muscle and silicotic nodule of guinea pigs and Croker sarcoma of mouse, and as seen in the figure, the activity of each tissue descreases in the order named.

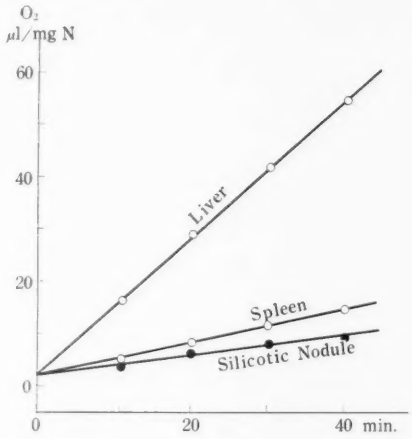

Fig. 3. Succinoxidose Activity of the Homogenate of Silicotic Nodule of Rat. (Schneider and Potter system)

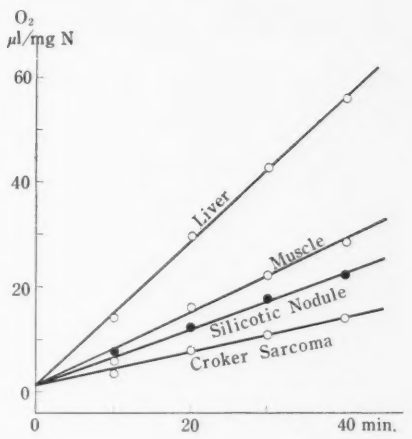

Fig. 4. Succinoxidase Activity of the Guinea-pig silicotic Granuloma. (Schneider and Potter system)

3) **Histological Findings.** Silicotic nodules of the rat's peritoneum were composed of histocytes, fibroblasts, reticulin fibers and weak collagen fibers, and the quartz particles were congromerated in the central area of the nodule (Fig. 5). But in the guinea pigs, the silicotic nodule was the granuloma composed of epithelioid cells and multinucleated giant cells, and there were not seen any fibers contrary to the case of rat's silicotic nodule (Fig. 6).

The rhodamine sarcoma is histologically a fibrosarcoma as originally reported by Umeda (5). The outstanding histological difference between rhodamine sarcoma and rat's silicotic nodule is the abundant fibroblast proliferation and poor fiber formation in the former.

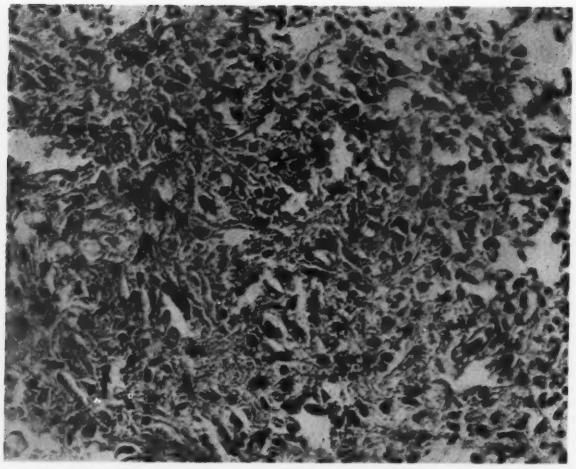

Fig. 5. Silicotic nodule produced on the omentum of a rat 6 months after the inoculation of quartz dust. The nodule consistes of histiocytes, fibroblasts and fibers. Haematoxylin and eosin staining.

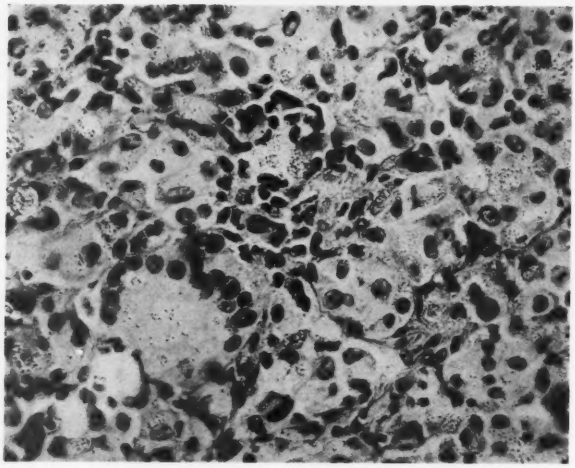

Fig. 6. Silicotic nodule produced on the omentum of a guinea pig after the inoculation of free silica dust. Epithelioid cells and multinucleated giant cells are clearly seen. Haematoxylin and eosin staining.

Histology of the Croker sarcoma is that of a round cell sarcoma, totally different morphologically from guinea pig's silicotic granuloma.

Discussion

Tissue reactions produced by quartz dust are different according to the species of experimental animals. In human, the silicotic nodule consists of collagen strands. Most active fibrogenic action of free silica is seen on the omentum of rats, but silica loses its fibrogenic action on the omentum of guinea pigs. These different tissue reactions against the free silica dusts may provide a key for the solution af the etiology of silicosis.

Rat's silicotic nodule contains abundant lipids (2). A German author (6) reported by electron-micrscopical investigations a morphological change of the mitochondria in the cells in reaction to cilica dusts, and from this fact, it was assumed that silica may have the inhibitory effect on the mitochondrial oxidation of the cells which phagocytosed silica dusts. In the present study, it was proved that the rat's silicotic granuloma has a lower respiration and higher fermentation than normal resting cells, and resembles to some extent the malignat tumor cells. Have silica dusts inhibitory effect on mitochondrial oxidation?

We have studied the effects of various silicic acids on the succinoxidase activities

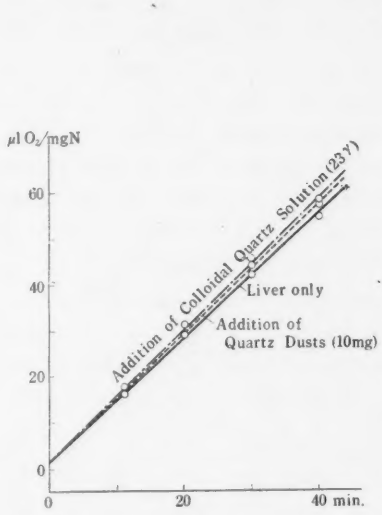

Fig. 7. Effect of Silicic Acids on the Succinoxidase Activity of the Homogenate of Rat-Liver.

Succinoxidase activity was assayed by Schneider and Potter's system. Quartz dust was the same as used in in vivo experiment. The colloidal quartz solution was prepared as follows: quartz dust was suspended in ammonia alkaline solutiion (pH 10.0) for 2 months with occasional agitation, after which the supernatant obtained by the centrifugation at 2000 r. p. m. for 30 minutes was heated to remove ammonia, and silicic acid in this solution was assayed colorimetrically after alkali-fusion.

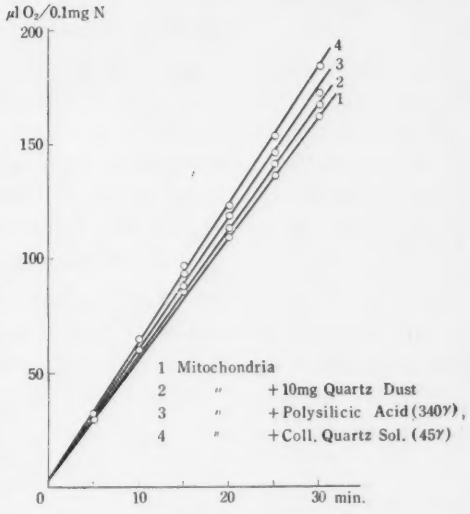

Fig. 8. The Effect of Silicic Acids on the Succinoxidase Activity of the Isolated Mitochondria of Rat-liver.

Succinoxidase activity was assayed by Schneider and Potter's system. The first vessel contained no silicic acid, the second 10 mg of quartz dust the third silicic acid which was prepared from sodium silicate solution by passing through ion exchange resin, and the fourth 45 r of colloidal quartz solution.

of the homogenate and isolated mitochondria of rat liver. These results are shown in Figs. 7 and 8. As seen in these figures, quartz dust, colloidal quartz solution, polysilicic acid show no inhibitory effect on the succinoxidase activity. Accordingly, it is hardly possible to assume that the respiration rate of mitochondria is lowered by the direct action of these silicic acids in short period. Relatively low respiration and high fermentation have been found in the rapidly growing cells and in the fibroblasts in tissue culture (7). Therefore, the metabolic pattern which we have found in rat's silicotic granuloma may be said to represent a character of growing histocytes or fibroblasts themselves. And it is very interesting that the same metabolic pattern was seen in guinea pig's silicotic granuloma, which is composed of epithelioid cells and multinucleated giant cells, as that of rat's silicotic granuloma, which is composed of histocytes, fibroblasts and fibers.

The relation between human silicosis and malignant tumor is a very interesting problem, but no significant relation has emerged statistically (8). But it was reported that the incidence of lung cancer among asbestos workers was high. In experimental animals we could not produce malignant tumors by silica alone, in the course of our silicosis study. Is there any way to convert into malignacy the character of silicotic nodule, which has a similar metabolic pattern to malignant tumor? In this connection, the report of Italian workers (9) may be instructive. They showed that rats injected with SiO_2 in the inguinal region on one side and with a mixture of SiO_2 and benzpyrene on the other developed tumors at the both sites of injection, suggesting the possiblity that silica may function as initiator or promotor to malignant tumor production, which must be studied further.

Summary

Some aspects of metabolic pattern of silicotic granuloma produced on the omentum of rats and guinea pigs were studied. Anaerobic glycolysis activity of the slices of rat's silicotic nodule was lower than that of rhodamine sarcoma but higher than those of muscle and liver. In guinea pig's silicotic granuloma, it was weaker than in Croker sarcoma, but stronger than in muscle and liver. Succinoxidase activity of the homogenate of rat's silicotic nodule was weaker than those of liver and spleen, and in guinea pigs, it was weaker than those of liver and muscle but stronger than that of Croker sarcoma.

Any silicic acid of quartz powder, amorphous colloidal silicic acid, and quartz solution had no inhibitory effect on the succinoxidase activity of the homogenate and of the isolated mitochondria of rat's liver.

References

1) Miller, J. W., and Sayers, R. R.: Public Health Rep., **49**, 80 (1934).
2) Sakabe, H., and Hirokawa, T.: J. Science of Labour (In Japanese), **28**, 789 (1952).
3) Warburg, O.: "Metabolism of Tumor" (translated by F. Dickens) Arnold Constable, London 1930.
4) Schneider, W. C., and Potter, V. R.: J. Biol. Chem., **140**, 217 (1943).
5) Umeda, M.: Gann, **47**, 51 (1956).
6) Schl, ipkoter, H. W.: Klin. Woch., **33**, 54 (1955).
7) Danes, B. S.: Exp. Cell Research, **8**, 543 (1955).
8) Schoch, H.: Z. f. Unfallmed. Berufkrankh., **47**, 138 (1954).
9) Carminati, V., and Chirio, M.: Atti soc. lombarda, sci. med. biol., **7**, 400 (1952) (quoted from Chem. Abst.: **47**, 7083 (1953).

要　旨

実験動物シリカ結節の代謝

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シリカをラッテ，モルモットの腹腔に入れ腹膜上に肉芽組織を作らせ，その代謝の様式を正常組織，実験腫瘍と比較してみた。

ラッテのシリカ結節の嫌気的解糖はローダミン肉腫には劣るが，正常の筋，肝よりは著しく高く，またモルモットのシリカ結節の嫌気的解糖は Croker Sarcoma より低いが筋，肝のそれよりは高い。コハク酸酸化酵素の活性はラッテ，モルモットの各結節ともに正常組織より著しく低いが，悪性腫瘍のそれを上廻っている。

粉末石英，コロイドシリカ，硅酸液のいずれも肝ホモヂエネート，ミトコンドリヤのコハク酸酸化酵素活性を試験管内では阻害しない。

これ等の所見からシリコーシスと悪性腫瘍の関係に考察を加えた。

[GANN, 48, 109~127; March, 1957]

PROCEEDINGS OF THE CONFERENCE ON BIOCHEMISTRY OF CANCER

On the occasion of the visit to Japan of Dr. Jesse P. Greenstein, Chief of the Laboratory of Biochemistry, National Cancer Institute, United States Public Health Service, a conference was held on the biochemistry of cancer on October 25, 1956, at the University Club, Tokyo, sponsored by the Cancer Institute, the Japanese Foundation for Cancer Research.

MEMBERS OF THE CONFERENCE

FUJII, TAKASHI. Zoology, Tokyo University
FUKUOKA, FUMIKO. Cancer Institute
GREENSTEIN, JESSE P. National Cancer Institute. (Special guest)
ISHIDATE, MORIZO. Pharmaceutics, Tokyo University
KONNO, KUNIO. Biochemistry, Tokyo University
MIURA, YOSHIAKI. Biochemistry, Tokyo University
MORI, KAZUO. Medical Zoology, Showa Medical College, Tokyo
NAKAHARA, WARO. Cancer Institute. (Chairman)
NAORA, HIROTO. Cell Research, Cancer Institute
ONO, TETSUO. Biochemistry, Cancer Institute
OOTA, KUNIO. Pathology, Cancer Institute
SHIMAZONO, NORIO. Biochemistry, Tokyo University
SUGIMURA, TAKASHI. Biochemistry, Cancer Institute
TERAYAMA, HIROSHI. Chemistry, Tokyo University
UGAMI, SABURO. Pharmaceutics, Chiba University, Chiba
YOSHIDA, TOMIZO. Pathology, Tokyo University
YOSHIKAWA, HARUHISA. Biochemistry, Tokyo University

Others present at the conference were: T. BABA (Cancer Institute), K. HARUNO (Showa Medical College), N. IIJIMA (Tokyo University), K. KURETANI (Scientific Research Institute), T. MIYAJI (Osaka University), M. SAITO (Cancer Institute), S. TAKAYAMA (Cancer Institute), M. TANAKA (Cancer Institute), T. TANAKA (Cancer Institute), M. UMEDA (Cancer Institute), K. WARABIOKA (Cancer Institute), and T. YAMAMOTO (Institute for Infectious Diseases).

Dr. Nakahara: To this group gathered together this afternoon, Dr. Greenstein needs no introduction. We all have known his name through his work, and through his masterly book "Biochemistry of Cancer", now for a long time. And, this afternoon, we have the rare good fortune of having with us this Dr.

Greenstein in person to participate in this conference. Dr. Greenstein arrived in Tokyo only yesterday afternoon, and disregarding his fatigue from travelling, has kindly consented to come to the meeting, and for this we are very grateful.

This conference is intended to be an informal one: no particular topic was chosen for discussion, and no fixed program was arranged. I have here a series of abstracts sent in from many of the Japanese members of this conference on what they wish to discuss. My job, as moderator, will be to try to organize them into some sort of shape as we go along.

Greenstein Law and Second Greenstein Law

Dr. Nakahara: With your permission, I wish to start the ball rolling by introducing what I propose to call The Greenstein Law, and The Second Greenstein Law in biochemistry of cancer.

Biochemical research on cancer has yielded several things which seem to contribute to our understanding of the nature of cancer. One of these is the concept of the biochemical uniformity of malignant tumors—the concept that cancer cells form a distinctive cell type biochemically. Regardless of the normal cell of origin, cancer cells tend to converge to a common biochemical pattern. This concept was developed largely by Dr. Greenstein, based not only on the observations of his own but also on a critical examination of data previously published by other authors. At a recent symposium in America, Weinhouse proposed to call it "The Greenstein Hypothesis".

"Hypothesis", however, does not seem to be the right word to use here. Hypothesis is after all a proposition or principle which is *assumed*. The convergence of biochemical patterns of cancer cells to a common type is not an assumption but is a generalization based on observed facts, and in my opinion, it may more properly be called a law, i.e., the Greenstein Law.

In close relation to the Greenstein Law, there is another thing which seems worthy of special consideration. That is the concept the cancer cells are capable of changing cancer-like the biochemical characters of the tissues of the cancer-bearing host.

In the first edition of his book, that is in 1947, Dr. Greenstein already clearly referred to this in connection with the now well known great decrease of liver catalase in tumor-bearing animals. "Tumors in a general sense", he said, "apparently present in themselves not only a rather uniform set of chemical components but also produce a more or less uniform effect on the host which contain them". He pointed out that the changes in the enzymatic pattern of the liver of the tumor-bearing animal are in the direction which the liver would take if it were transformed into a hepatoma. This idea of generalized and systemic

"cancerousness" of the tissues of tumor-bearing animals, I now propose to call the Second Greenstein Law.

In a way this Second Law is subordinate to the First Law, since the phenomena taking place according to the second law depend upon one or another of the properties common to all the cancer cells. But the second law is not a part of the first. since it deals with the effects which cancer cells produce on the host, while the first law is concerned with cancer cells themselves.

How many different organs and tissues, besides liver, are involved in this Second Greenstein Law cannot be stated at this time. There are, however, evidences which indicate other tissues than liver also behave according to the law. For example, Boyd and associates at Queen's University recently showed that in lipid and water contents diaphragm, oesophagus, intestine, etc., of the tumor-bearing host gradually aquire that sameness of composition which characterizes the tumor.

Dr. Fukuoka: I am doing some screening tests in experimental cancer chemotherapy, and, like other people working in the same field, I am impressed by the different reactions of different tumors to a given chemical agent. I do not believe, however, that that contradicts the Greenstein law. The Greenstein law simply states that cancer cells, regardless of the cell of their origin, tend to converge to a common biochemical pattern. It seems to me perfectly reasonable that different tumors may be different in various minor particulars and yet agree as to some important biochemical characters. It may be like all the species belonging to one genus agree as to the generic characters and still have their own specific characters.

Dr. Greenstein: I am deeply grateful for this opportunity of discussing problems of our mutual interest with this distinguished Japanese group, and especially appreciate the great courtesy on your part in carrying on the conversation entirely in English. Dr. Nakahara has lucidly presented the concept of the biochemical uniformity of cancer tissue, but I must gratefully but firmly repudiate this suggestion of any law associated with my name. It is a generalization based on the work of not one man but of many, including Dr. Nakahara himself.

The problem of whether cancer tissues converge to a common biochemical pattern is no longer a theoretical one. It is a concept based on findings in the laboratory. This has been challenged. We find the field of cancer research divided into two camps, people working in the laboratory finding uniformity, with clinicians taking the position that cancers are diverse and not identical. This comes down to the contrast between laboratory workers investigating the chemistry of cancer tissues and clinicians observing the behavior of tumors in

the living patient. Chemists and clinicians see things differently. We must try to understand each other, but we must be sure we are using the same language.

No one did more work than Dr. Nakahara, Dr. Fukuoka and their group on the enzyme control mechanism in tumor-bearing animals, especially the liver catalase level. The toxohormone concept is to be fitted into the whole picture in animals. This is a study of enzyme-controlling factors arising in the body of living animals and regulating the level of its enzymes. The choice of term "toxohormone" by Dr. Nakahara and Dr. Fukuoka is a happy as well as pictorial one. It is a circulating factor and the term expresses the thought very well indeed.

As said by Dr. Fukuoka, many chemical agents used in chemotherapy act differently to different tumors. The reasons for this may become clear as we get to know more about the tumor-host relations.

However, I must say that I came to Japan not to talk but to listen.

Dr. Nakahara: The topic having been brought to toxohormone, I shall ask Dr. Ono to speak on some of his newer findings.

Toxohormone

Dr. Ono: I would like to show you the most potent preparations of toxohormone which have been obtained in our laboratory.

At the first step, the alcohol precipitate of boiled tumor extract was prepared by the procedure of Dr. Nakahara and Dr. Fukuoka. And it is effective in 50-100 mg doses in the mouse in depressing liver catalase activity, as widely confirmed. Then this fraction was subjected to methyl alcohol-acetic acid (3 : 2) extraction, and 20 percent of the starting material was recovered by this procedure. The nucleic acid contamination in the starting material could be eliminated almost completely, and fortunately all of the activity in the original preparation was concentrated in this fraction. As a result, this fraction was active in 10 mg dose per mouse, and 5-10 fold concentration was attained by this procedure, and was revealed to be composed of polypeptide almost entirely.

For the further purification, alcohol fractionation, zone electrophoresis, and ion exchange resin colum chromatography were applied, but no definite results could be obtained. In these trials, the cellulose powder column chromatography yielded appreciable good results, and in the case of Murphy lymphosarcoma we got a fraction effective in as small as a 0.5 mg dose by this procedure.

As confirmed by Dr. Nakahara, pepsin digestion resulted in no inactivation of toxohormone, so we attempted to carry out the fractionation of the pepsin digest by the procedure of Felix et al. for the fractionation of pepsin digest of histone. The procedure was as follows: Methanol-acetic acid extract of raw toxohor-

mone was incubated with pepsin, 1 percent of its weight, in 0.1 N HCl for four days at 37°C under toluene. At the end of the incubation it was boiled for 5 minutes and centrifuged. Then the reaction of the clear supernatant was adjusted to pH 8.3, and saturated Na-picrate solution was added at as much as 1/3 of its volume. In the course of 30 minutes after the addition of picrate, a precipitate with a crystalline sheen gradually appeared. By microscopic examination it was revealed to be composed of fine needle-shaped crystals. Its activity was assayed after removal of the picric acid moiety, and revealed to be active in 0.1–0.2 mg dose.

As to the homogeneity of the preparation we have no conclusive data as yet, but, it showed a single spot by paper chromatography with the two different solvent systems. The amino acid composition was determined by the method of Levy et al., and revealed to contain 13 amino acids, exclusive of cystine, tyrosine, tryptophane, and methionine. The smallest unit of this peptide consists of about 32 amino acid residues, and its molecular weight was estimated to be about 4000. At the present time, we do not think that it is the final preparation of toxohormone, and we are now seeking procedures leading to a purer preparation.

Dr. Greenstein: How many basic amino acids are present in the picrate? How much picric acid forms the salt: and to one molecule of toxohormone how many molecules of picric acid are present?

Dr. Ono: Arginine and lysine are present. The nitrogen content of the picrate is about 13 percent; that is Kjeldhal nitrogen.

I reiterate that we do not consider this crystalline picrate as the final preparation. We are seeking further purification.

By the way, recently we have succeeded in demonstrating the presence of toxohormone in the culture medium of HeLa cells, which is accepted to have originated from human uterus carcinoma cells. Up to this time, two preparations were obtained from several days old culture media which had contained about several million cells, by adopting the benzoic acid absorption method as established by Dr. Nakagawa.

The preparations obtained are fairly active in 25 to 50 mg dose, while the same fraction from the control culture medium in which cells have not been cultivated exhibits no effect. It is hoped that these experiments may offer a useful clue to examine the malignancy of cultured tissue.

Dr. Nakahara: This is a clear evidence that toxohormone is a product of proliferating cancer cells. It is remarkable that human cancer cells continue to produce toxohormone after so many thousands of transfers in tissue culture.

Dr. Greenstein: If I may say so, this presentation of Dr. Ono is not only very lucid but is of great scientific importance. May I ask if at any time was a test made on crystalline catalase *in vitro*?

Dr. Ono: Tests were made always *in vivo*, because toxohormone is known to be active only *in vivo*.

Dr. Nakahara: This a good chance to ask Dr. Sugimura to present his work on the so-called *Kochsaft* factor, which is active on crystalline catalase *in vitro*.

Dr. Sugimura: In 1951, Drs. Nakahara and Fukuoka suggested that an impaired synthesis may be the cause of the liver catalase depression of tumor-bearing animals. On the other hand, Drs. Hargreaves and Deutsch reported in 1952 that tumor *kochsaft* directly inhibited the catalase activity *in vitro*, using crystalline catalase. We considered it important to clear up the relation between the toxohormone of Drs. Nakahara and Fukuoka and the so-called *kochsaft* of Drs. Hargreaves and Deutsch.

We could confirm the existence of Drs. Hargreaves and Deutsch's tumor *kochsaft* factor by our experiments using many transplantable tumors. But at the same time, it was also demonstrated that the boiled extract (*kochsaft*), prepared from normal liver, spleen, kidney, embryo and other tissues, brought about similar degrees of inhibition.

Furthermore, we found that glutathione inhibits crystalline catalase after *in vitro* incubation at the temperature of 0°C. Then, we determined the glutathione content in *kochsaft* of normal and tumor tissues. In view of these facts it is obvious that a portion of the inhibiting action of boiled tissue extract may be caused by the glutathione contained in it.

On the other hand, toxohormone, obtained by Drs. Nakahara and Fukuoka's method, does not inhibit crystalline catalase *in vitro*.

We can safely conclude that Drs. Hargreaves and Deutsch's *kochsaft* factor is entirely different from toxohormone which acts only *in vivo*. *Kochsaft* factor is non-specific to tumor tissue.

Dr. Greenstein: What strikes me most vividly is that a considerable degree of inhibition of crystalline catalase is brought about by so many different *kochsafts*. Deutsch found the inhibitor only in spleen and liver. Your *kochsafts* were from normal animals, not tumor bearing, and they showed inhibition of the same order of magnitude as that of tumor tissues. Some anticatalase must exist in normal tissues. Did you determine the catalase activity only by the splitting of H_2O_2 or use spectroscopic criteria also, as did Hargreaves and Deutsch? It is a happier arrangement if experiments were done with the both methods. Dr. Sugimura's work casts a considerable doubt on the concept of Hargreaves and Deutsch.

Dr. Sugimura: We did not make spectroscopic examinations. Hargeaves and Deutsch said that the *kochsaft* factor is present only in malignant tumor based on the measurement of the remaining H_2O_2, so it seemed only neccessary to examine the activity in repeating the work. Our question was whether *kochsaft*

factor is specific to tumor tissues or not, and Ceriotti has arrived at the same conclusion as ours.

Dr. Nakahara: Do you think, Dr. Greenstein, that a sufficiently purified toxohormone could possibly inhibit crynstalline catalase *in vitro*?

Dr. Greenstein: I thought, Dr. Nakahara, that one of the points you made was that toxohormone was only a part of a larger compound or system which acted *in vivo*. Did you not say that?

Dr. Nakahara: What we previously reported was that toxohormone comes in smaller, dialysable form as well as in aggregates of such forms or combined with other substance.

Dr. Greenstein: What you succeeded in doing, as has Dr. Ono recently, may have been to split off an active fragment, like one splits off hematin from hemoglobin, the fragment which becomes reactivated when put back into the animal body, the active prosthetic group which becomes reactivated *in vivo*.

Crude *kochsaft* is just a mass of material, which *in vitro* would produce a great many non-specific reactions. The process of purification apparently removes some cofactor which is supplied by the whole animal, and which is present in *kochsaft* together with toxohormone.

Purification is a delightful if difficult task. It certainly gives an aesthetic pleasure, but, perhaps it may take one further away from the biological complex which actually works.

Dr. Terayama: I had been studying the *in vitro* inhibition of purified catalase by polyelectrolytes. Catalase was inhibited remarkably by various kinds of native and synthetic polyelectrolytes including nucleic acids, mucopolysaccharides, etc. The inhibition by these substances was dependent on the pH of the medium as well as the concentration ratio of the two components. Apparently this kind of inhibition is not specific to catalase. I just recall these former experiments of ours in connection with the experiments of Hargreaves and Deutsch as well as of Abrignani and Mutolo.

Dr. Greenstein: It is very interesting. I think we have to prepare a complete list of catalase inhibitors having various physicochemical characteristics. Many substances active *in vitro* must be considered separately from biologically produced substances. These are questions of chemistry, not of cancer. There are many non-biological substances which strongly inhibit catalase.

Dr. Nakahara: Dr. Sugimura has been working on one of just such substances.

Dr. Sugimura: Last year, Dr. Heim and others reported that the injection of 3-amino-1, 2, 4-triazole into rats causes a remarkable depression in liver catalase but not in blood catalase. They pointed out that this effect of AT was similar to that of malignant tumors. So, we tried to clarify the mode of action of AT

as related to the mode of action of toxohormone.

We could not only verify the results of Dr. Heim's experiment but also many interesting problems about AT inhibition emerged into view.

A remarkable liver catalase depression was produced by the injection of AT.

In *in vitro* experiments of mouse liver homogenate using AT, the final concentration of AT was one milligram per millilitre. With the incubation at 37°C, catalase activity was almost completely inhibited but with the incubation at 0°C catalase was not inhibited at all.

In experiments using crystalline catalase at 37°C, contrary to the data obtained from the homogenate experiments, crystalline catalase solution was not affected by AT. When liver extract which is obtained by centrifugation at 23,000×g was added to crystalline catalase and AT mixture, the activity was completely lost. With liver extract alone crystalline catalase can be inhibited, but to a lesser degree only.

We carried out some experiments with liver extract. Liver extract was boild or dialysed, and it was found that boiled or dialysed liver extract cannot develop the AT action. When two extracts were combined, the inhibitory action of AT was completely restored. In conclusion, it is evident that the liver extract is composed of two components, namely a heat-labile, non-dialyzable protein, and a heat-stable, dialysable cofactor. The cofactor can be replaced by compounds containing purines.

In experiments under aerobic and anaerobic conditions, it was made clear that AT inhibition on liver homogenate catalase does not appear under anaerobic conditions.

From the data presented, it is clear that for the complete inhibition of catalase, it must be incubated together with AT and liver extract containing some protein and cofactors at 37°C under aerobic conditions.

Furthermore, we could not find an inhibition of cytochrome c oxidase activity, or a disturbance of porphyrin metabolism by AT *in vivo* or *in vitro*.

These results seem to indicate that the action of 3-amino-1, 2, 4-triazole on catalase is really quite different from that of toxohormone.

Dr. Greenstein: Do I gather that all the activity of crystalline catalase disappears irreversibly?

Dr. Sugimura: Yes, almost completely, and irreversibly. Dialysis does not restore the activity of catalase.

Dr. Greenstein: The essential problem is whether toxohormone and/or larger complex, of which it is a part, reduces liver catalase activity by combination or by inhibiting the synthesis of catalase. *In vitro* experiments are interesting in showing chemical interraction, but there is the possibility that less catalase is

synthesized in the sedormid effect, and that the synthesis of catalase is interfered with in some manner.

Dr. Nakahara: We have evidence to show that toxohormone interferes with porphyrin metabolism, which speaks in favor of the idea that toxohormone inhibits liver catalase synthesis.

Dr. Ono: As to the mode of action of toxohormone and/or the decrease of liver catalase of tumor-bearing animals, Dr. Nakahara and Dr. Fukuoka have suggested that the synthesis of iron-porphyrin enzyme may be impaired probably by the disturbance of iron and protein metabolism. If that is the case, it may be reasonable to expect a porphyric state in tumor-bearing animals, as observed in experimental porphyria caused by lead or Sedormid. Concerning Sedormid, it has been revealed by Schmid and his colleagues that it produces a marked fall of liver catalase and a severe porphyric state ***in vivo***.

The results of our experiments indicate a considerable increase of the porphyrin content in the blood, liver and urine of the tumor-bearing rat. In toxonormone-injected animals, a similar increase of liver protoporphyrin contents was also noted.

But in the synthetic activities of porphobilinogen and protoporphyrin from 3-aminolevulinic acid, there was no difference between normal rat liver and that from the tumor-bearing animals. Thus, we may conclude that the relatively high levels of porphyrin in tumor-bearing as well as in toxohormone-injected animals resulted not from the hyperproduction but from the underutilization of porphyrin, which is brought about secondarily by the injury of iron and protein metabolism of hemoproteins.

This conclusion is compatible with our hypothesis about the action of toxohormone.

Dr. Greenstein: Did I understand correctly that there was no increase in erythrocyte porphyrin? Is this purely in liver?

Dr. Ono: Protoporphyrin in blood is increased to twice the normal amount.

Dr. Greenstein: Yet there is no change in the catalase level in erythrocytes.

Dr. Ono: We can perhaps explain that by the longer life span of erythrocyte catalase, which is more that twice that of liver catalase, according to Theorell.

Dr. Greenstein: I like to raise some questions. We do get in tumor-bearing animals a lowered blood cell number: there are less red cells, but hemoglobin is very substantially diminished. Where does this hemoglobin go to? In tumor-bearing animals due to some form of destruction of red cells hemoglobin may go to the tumor. Animals given toxohormone do not show lower hemoglobin. Is that true?

Dr. Nakahara: A single injection of toxohormone does not affect hemoglobin,

but repeated injections eventually lower hemoglobin.

Dr. Greenstein: Effect of toxohormone injection is of short duration, whereas the tumor-bearing animal has the tumor for a long time. Does the destruction of red cells and hemoglobin have anything to do with the lowering of catalase?

Dr. Nakahara: The lowering of catalase takes place long before that of red cells and hemoglobin in animals treated with toxohormone.

Dr. Greenstein: Toxohormone is a means of simply speeding up or increasing the rate of what might be called the tumor effect. We are doing in one shot what the tumor may accomplish in a month.

Biochemical Study of Cancer in Japan

Dr. Nakahara: Toxohormone seems to have received more than its share of discussion. By way of introducing other subjects, I may briefly touch upon the present situation of biochemical studies on cancer in Japan.

Cancer research in Japan, very much more so than in the United States, has been dominated by pathologists, and even today, the number of biochemical studies are incomparably less than pathological ones. I do not mean to say that there are too many pathologists. We can never have too many workers in any field. What I wish to see is many, many more biochemists working on cancer.

In the post-war Japan there has been a gradual rise of interest in the biochemistry of cancer. I am bound to say that this rise of interest is to no small measure due to the publication of Dr. Greenstein's "Biochemistry of Cancer". The first edition of this book was translated into Japanese ***in toto***, and that is an eloquent testimony of what I am saying.

This year, eleven years after the war, we have come to organize a special research group or committee on the biochemistry of cancer. The Japanese Government Ministry of Education offers grants-in-aid for similar research groups in various fields of science, and this cancer biochemistry group was organized as one of such groups.

Fourteen workers officially compose this group at present, and although the amount of grants allocated to us is not sufficient I believe that it is a good start.

I may enumerate the major topics which are being taken up for study by this research group. That may give an idea as to the type of studies that are being emphasized in Japan at the present time.

1. Isolation of various proteins composing cancer tissue, and elucidation of their structures and biochemical functions. This is an ambitious program undertaken by Dr. Akabori.

2. Investigation of the metabolic mechanism of nucleic acid, especially DNA, with special reference to their relative metabolic stability. Drs. Miura, Mizuhara,

Naora, Sibatani and Ugami are participating in this aspect of the studies.

3. Studies on toxohormone, especially in relation to abnormal metabolism of cancer cells and of cancer-bearing hosts. Drs. Masamune, Ono, and Suda are working in this field. Dr. Konno's work on heme protein may converge on this subject.

4. Enzymological studies, including enzymatic changes in liver tissue during the course of its malignant transformation. This is largely a continuation of an old theme, by Drs. Kishi, Mizuhara and Mori.

5. Significance of zinc in cancer tissue. This subject is being investigated by Dr. Fujii in connection with broader studies on mitotic mechanism.

The group meets twice annually to report on the progress of work and to informally discuss the problems under investigation. The chairman is responsible for the conduct of the group to the Ministry of Education. This responsibility is mine for the time being.

Biochemical Changes in the Course of Carcinogenesis

Dr. Nakahara: Changes in the enzymatic activities of liver of rats in the course of liver cancer production is a subject that has been under investigation for the longest period. Perhaps Dr. Mori might give a summarized account of it.

Dr. Mori: For the past several years, attempts have been made to establish a relationship between the enzymatic activities and the liver tissues of rats during the course of liver cancer production which have been reported from the Laboratories of Biochemistry and Medical Zoology, Showa Medical School.

After feeding the rats with p-dimethylaminoazobenzene or 2-acetylaminofluorene routinely so as to produce liver cancer, feeding was continued for additional several weeks with the basal diet free from the carcinogen. Then, the animals were sacrificed and livers were classified macroscopically into four groups: slightly and moderately hyperplastic, cirrhotic and cancerous. These liver findings represent various grades of pathological changes leading to the production of liver cancer.

Twenty-one enzymes of the whole liver homogenates were studied. In general, the two carcinogens, p-dimethylaminoazobenzene and 2-acetylaminofluorene, induced almost a similar result in the content of the enzymes in the hepatic tissues and in the histological changes. The relative activities of the enzymes studied are shown in Table 1. As shown in Table 1, three types of changes in the activities of the enzymes were found in the hyperplastic livers. Namely, when the liver tissue changed into hyperplastic the activities in some enzymes become higher, or lower than that of the normal, while others remained unchanged. When the liver tissues turned into cancerous, the activity of some enzymes

Table 1

Enzymes	Relative Activity*			
	Hyperplastic		Cirrhotic	Cancerous
	Slight	Moderate		
Deaminase of Fatty Acid Amides	98	89	59	5
Asparaginase	85	87	74	14
Choline Oxidase	90	60	68	17
RNA Deaminase (pH 11.0)	112	130	149	218
Arginase	91	104	91	17
Acid Phosphatase	93	90	90	69
Acylase (Acetanilide)	112	110	95	0
Succinic Dehydrogenase	100	92	98	68
Monoamine Oxidase	89	100	113	49
Glycolate Oxidase	99	110	74	35
Esterase	107	98	100	54
Cholinesterase	100	102	144	1170
Glutaminase	112	148	116	258
Catalase	100	113	49	14
Uricase	125	114	94	23
Rhodanese	125	105	91	13
Cathepsine	166	136	119	82
Acylase (Diacetyltyrosine)	124	172	152	118
RNA Deaminase (pH 5.0)	123	112	114	100
Guanase	91	71	82	97
Alkaline Phosphatase	56	49	84	566

* Value for normal liver is taken as 100.

reached its lowest or the highest level. Some enzymes in the cancerous tissue kept their activities in normal degree. There are eight types of the changes in the enzymatic activities of liver during the carcinogenesis. If we represent the various enzymatic activities in normal rat liver schematically in the shape of a doughnut, it is clearly seen that the shape of doughnut becomes irregular as the pathological changes progress.

Whether or not the series of changes in the enzymatic activities are responsible for the production of liver cancer remains an open question, which can only be solved by further studies.

Dr. Greenstein: Did you use centrifuged extracts or the whole homogenate? You find glutaminase very high in tumor tissue. There are two kinds of glutaminase, one insoluble and activated by phosphate and the other soluble and activated by pyruvate. Which glutaminase was studied?

Dr. Mori: We used whole homogenates. The glutaminase studied was of the phosphate-activated kind.

Dr. Greenstein: The amount of enzyme may depend on the number of mitochondria, and there may be cases where there is more enzyme simply because there are more mitochondria. Enzyme-increase per unit nitrogen may be regarded as a specific increase. Increase or decrease of enzyme must be related to the amount of particles. In tumor tissues, in spite of decreased mitochondria, there is increased glutaminase, and that is very interesting.

Dr. Ugami: The following problems are as yet far from being clear, but I would greatly appreciate if Dr. Greenstein would tell us his opinion.

Though a large number of enzymes have been studied as to their changes in tissues during carcinogenesis, what enzyme system or pattern is most closely related to the action of chemical carcinogens? In other words, what is primarily attacked by chemical carcinogens?

What do you consider is the mechanism or processes under which the above-mentioned changes of enzymic pattern develop into the so-called cancer-characteristic and irreversible pattern of enzymes?

In view of studies, for instance, of Schneider or Butenandt, who demonstrated the cytoplasmic changes in cancer or precancerous cells, do you think of the cytoplasmic constituents as the primary attacking point of carcinogens? If so, what do you consider the significance of the nucleus or nucleolus in the precancerous stage to be?

Dr. Greenstein: What exact cellular compound is affected will depend upon the real carcinogen, not necessarily the substance used as carcinogen. The most obvious clue may be the attachment to some protein, which combines with carcinogen or its metabolic products. This is soluble, therefore, a cytoplasmic component.

Dr. Terayama: Recently Mr. Kusama and I have started to separate the protein or proteins which may combine with DAB or its derivative in rat liver cells. We have not done much on this problem yet, but, it seems to me that it might be of a globuline-like nature. We are now trying to purify the protein components in the soluble state by alcohol and ammonium sulfate fractionations. At the same time we are conducting some experiments on the nature of the binding of DAB with the protein. Cysteine or tyrosine may be responsible for the binding.

Dr. Greenstein: Tryptophan could be responsible. What kind of biological activity do you think the DAB binding proteins may have?

Dr. Terayama: That is what we want to know most. We hope they may be some oxidative enzymes.

Dr. Oota: I am afraid that my point may be little too practical to discuss on this occasion. But, as one of the pathologists, I have been much concerned with

the early recognition of cancer, and what I feel now is that we are confronting an almost unbreakable barrier in this problem.

Even in experimental carcinogenesis, it is extremely difficult, if not impossible, to recognize the earliest phase of cancerization. With Rous, Berenblum, and others, we may postulate a three-step process in cancerization, say of the liver, skin or any other kind of tissues. I am referring to the stages of so-called precancer, initiation and promotion. Who has seen the exact moment of initiation, and who can tell a cancerous from precancerous cells *in situ*? In spite of many futile efforts we have not even been successful in establishing the criteria of malignancy in the field of morphology. The only objective clue for the early recognition of experimental malignancy appears to be in its transplantability, i.e., its biological behavior as Dr. Greene puts it, and which is, as you know, so precarious on experimentation.

As I understand, a solution of this problem from the biochemical aspects is rather on a statistical basis than on a basis of analysis of various activities of a small group of cells or a single cell, so far as the biochemists are dealing with the collective materials which are supposed to be malignant, benign or "precancerous", and so labelled by morphologists.

One of the intrinsic difficulties of that type of studies is that you have no histological control of the same material you chemically analyze. Recently, Weiler and Butenandt from Germany, utilizing the immunohistochemical method of Coons, claimed to have succeeded in the histological visualization of the malignant transformation of hepatic cells in experimental carcinogenesis of rat liver. At present, however, this technique is not applicable to human materials.

In dealing with the so-called carcinoma *in situ* of the human uterine cervix, we tried to find some direct correlations between the biochemical behavior and morphology of the lesions under question. About 10 mg of the scrapings of a suspicious lesion, located by means of a colposcope, was put into a Warburg manometer with a special small capacity, and its glycolytic activity was assayed by classical methods. After completion of the procedure, the tissue material was freeze-dried, and embedded in paraffin under vacuum. The dry-weight of the material was obtained, and the glycolytic activity was given in relation to the dry weight. Serial sectioning of the paraffin block so prepared revealed an excellent material for histological checking of the whole tissue which had been examined biochemically. A diagram obtained from a series of such histologically debatable lesions shows a good parallelism of the intraepitherial tissue atypism and glycolytic activity. An adjustment on the basis of an exact count of cell number may be feasible. However, we have not yet succeeded in correlating such biochemical activity with atypism of a single cell or of a small group of cells.

Naturally, we are not the first to assay that kind of activity of human carcinoma *in situ*, but previous workers failed to be exact in the histological control, because they did not use a freeze-drying technique, and only tried to examine the adjacent and not the same tissue material.

We are fully conscious that high glycolytic activity is not specific to malignancy, and we would very much appreciate your suggestion along this line of research, since a collaboration of biochemists and pathologists is essential to the solution of the problem of early recognition of cancerization. The diver technique may be useful for a smaller quantity of tissue material. We are even thinking of assaying, somehow, the activity of a small amount of human and animal material in synthesizing the toxohormone.

Dr. Nakahara: In studying chemical changes in tissues biochemists are working on large masses of tissue on the average basis. Studies on per cell basis are needed.

Dr. Greenstein: That is the problem of microchemistry. The finer manipulation is an art, but most chemists are not familiar with it.

Dr. Oota: What do you think of the use of tissue culture for such purpose?

Dr. Greenstein: In using tissue culture the possibility of changes in the character of cells during many transfers must be considered. The action of selection may give quite a different type of cells from the original cell population.

Dr. Nakahara: What Dr. Oota needs is a supermicrochemical method. I said "cellular basis", but we have studies on zinc by Dr. Fujii on the "nucleolar basis".

Zinc in Nucleoli and Chromosomes

Dr. Fujii: We have already reported that the nucleolus contains a high concentration of zinc and that the chromosomes also appear to become laden with zinc on the initiation of mitosis. Zinc could be detected simply by adding a small amount of alcoholic dithizone to living cells. The proof that zinc is responsible for this dithizone reaction came from observations on polarograms. When the ash of the isolated nucleoli of the starfish oocytes, *Asterias amurensis*, was analyzed polarographically, it was found that the zinc content of these nucleoli was 2.6 μg/mg dry weight, on the average. This was further definitely confirmed by experiment using Zn^{65}. The content of this isotope was found to be ten times as high in the isolated nucleoli as in cytoplasmic fractions (plus nuclear debris), when radioactivity determinations were made after the starfishes had been exposed to Zn^{65}.

Furthermore, it is extremely interesting to observe that the chromosomes and spindle of the eggs of the sea-urchin, *Clypeaster japonicus*, become dithizone-

positive on entering the mitosis, the chromosomes then becoming reversibly colorless as they swell in the telophase. Another significant fact is that the spindle becomes suddenly colorless with the start of anaphase. From these and other observations I am inclined to believe that zinc is involved in the mechanism of mitosis.

Now, the question naturally arises as to whether the behavior of zinc is also related in some way to the problem of cancer. Nuclei were isolated from rat hepatoma and normal rat liver and their zinc content was determined. It was found that the zinc content of hepatoma nuclei was always higher than that of normal liver nuclei, the values for the former being 0.18 μg/mg dry weight and for the latter 0.08 on the average. The cytoplasmic fractions of the tumor tissue generally contained a little more zinc that did the fractions from the normal tissue. It was also found that zinc occurs in these tissues largely in association with a lipoprotein, which is essentially the same substance that Stedman and Stedman called "chromosomin" (cf. Wang, Mayer and Thomas, 1953). The carcinogenic p-dimethylaminoazobenzene was also found to occur constantly combined with this nuclear protein in the early stages of the feeding. The zinc content of this fraction increased considerably during the course of liver cancer formation, although the amount of the lipoprotein decreased during the same period, and instead, another protein (precipitable at pH 4.0) was greatly increased in amount only in the case of tumor nuclei. It is very striking that chromosomes and nucleoli when they are treated with dithizone are more deeply stained and appear in greater relief in the hepatoma nuclei than in normal nuclei.

There is some evidence that zinc is firmly bound to proteins in hepatoma tissue than in normal tissue. Therefore, we may assume tentatively that a certain change in the nature of the zinc-protein linkage is, in part, responsible for the neoplastic transformation of cells, these changes interfering with the normal, finely coordinated behavior of the zinc during the mitotic sequence.

Dr. Greenstein: Some enzymes are associated with zinc. The increased zinc in nucleoli may have some enzymatic implication.

Dr. Naora: Is the stained zinc protein directly handed down to daughter cells when the cell divides?

Dr. Fujii: Yes, the color is distinctly visible during the entire process of mitosis.

Dr. Greenstein: If the cancerous process is associated with increased amount of zinc, what would be the effet of decreasing the zinc intake? It may be possible, though remotely, to decrease the zinc intake not enough to interfere with life but sufficient to prevent cancer cell proliferation. The excretion of zinc in the urine may be due to some form of decomposition of zinc protein. It is all very interesting.

Concerning Nucleic Acid

Dr. Nakahara: No conference on biochemistry can be complete without some reference to nucleic acid. We have two speakers in this field.

Dr. Miura: I wish to present an outline of the study on the metabolism of nucleic acids in human carcinoma. The mammary tissues of fifteen cases of human mastopathy and mammary carcinoma were removed by surgical operations. The tissues were incubated ***in vitro*** with P^{32} at 37°C for 2 hours. The lipid fraction and RNA fraction were extracted and their relative specific activities were compared in two groups: mastopathy ***versus*** carcinoma.

It was found that the amount of phospholipids was higher in the tissue of carcinoma than mastopathy. The relative specific activity of RNA was also higher in the case of carcinoma than mastopathy. It was also found that the previous administration of male hormone decreased the relative specific activities of RNA both in mastopathy and carcinoma.

Dr. Naora: It is accepted that the dexypentose nucleic acid is an important cellular component which must be considered in investigations of the chemical nature of the multiplication of cells, but the question as to how it works in a cell remains obscure. Although there are a number of investigations concerning the metabolism of nucleic acids in tumor cells, I wish to discuss whether we may assume that DNA is metabolically stable in tumor cells, and whether its content in an individual cell is constantly maintained as in the case of normal cells.

First, I would like to speak of the stability of the DNA molecule in living cells. Generally the uptake of various isotopic precursors by DNA in tissues is paralleled by the mitotic rate, but not in non-dividing cells. Moreover, no release of isotopes from previously labelled DNA was observed by some workers on different materials including proliferating tissues, such as the liver of suckling rats, regenerating liver, cells of Earle's L strain, Ehrlich carcinoma, etc. From all the results which are available, it seems almost certain that in both animal and bacterial cells DNA is replicated without any breakdown of old molecules of DNA in the DNA synthesis before the cell division. At present, Leblond's hypothesis reported in 1953 does not hold even in neoplastic tissues. Such stability of DNA in the living cell is not due to the physical stability of DNA itself, but to the organization of the living cells. It is clear that DNA usually does not remain intact in dead cells for a long time.

Secondly, I would like to speak of the maintenance and constancy of DNA content. Since the pioneering efforts of Boivin and his collaborators, the quantitative investigations of the constancy of DNA content per nucleus have been repeatedly attempted by using both the biochemical and microspectrophotometric

techniques. According to my experiments by the technique of microspectrophotometry, integration of the results shows that as far as the different tissue cells of one and the same animals are concerned, the constancy of the DNA content per nucleus can be maintained. However, the DNA content per nucleus of a given ploidy class is subject to variation which may possibly be related to the course of postnatal growth.

In investigations of the amount of DNA per nucleus of tumor cells, it may be accepted that the constancy of the average DNA content per nucleus can be demonstrated, as cited by Dr. Greenstein in his book, Biochemistry of Cancer. We often found the increased amount of DNA in tumor cells. But such an observation may be partly explained as due to the appearance of polyploid cells and of the cells which are synthesizing the DNA molecules. In a number of studies by the techniques of microspectrophotometry, some deviation of the DNA content per nucleus from the mean value and the appearance of hyperploidy indicate that some cells, having an amount of DNA apart from the mean value, possess some accerelated activity of DNA synthesis and are lacking in the close correlation between DNA synthesis and mitosis: moreover, most of the tumor cells appear to contain an organization to maintain the normal DNA content. However, such character is not necessarily specific for tumor cells, because it is known that the mechanism of DNA synthesis is not directly linked with the mitotic mechanism in normal cells.

From all results, it seems certain that the biochemical character of tumor cells with respect to the stability and the maintenance of DNA is not abnormal. It may be considered that tumor cells are representative of cells having the most significantly accelerated activity of cell-multiplication, including that of DNA synthesis. Therefore, we may compare the stability and maintenance of DNA in tumor cells with those of normal cells without making any appreciable distinction between normal and tumor cells.

With such considerations, it would seem interesting to refer to the *in vitro* investigation of DNA-maintaining systems. Such studies are based on the measurement of the decreased amount of the DNA content in cells incubated *in vitro* in a suitable medium, for example Krebs-Ringer solution at pH 7.0. An experiment was performed using liver cells having various degrees of DNA synthesis, such as liver cells of the adult rats weighing about 200 or 300 g in body weight, the young rat weighing 20 to 40 g in body weight, cells of the regenerating liver, embryo, and transplantable ascites hepatomas. The raw data were converted into relative values by taking the initial value of the average DNA content per cell in the tissue in question as the reference standard. The results showed that in liver cells of adult rats, the DNA content per cell gradually

decreased. But, in the hepatoma cell, we could not observe such decrease of the amount of DNA. The result of this investigation shows that there is a strict correlation between the abilities of DNA-maintaining systems and the DNA-synthesising processes.

Dr. Greenstein: Does RNA show a similar picture, substantially? And is the stability judged by the incorporation of P^{32}?

Dr. Naora: RNA shows a more significant decrease during incubation. The stability was judged by the release of nucleic acid as determined by the usual chemical procedure.

Dr. Greenstein: The enzymes that affect DNA may be different in tumor from normal tissues, or DNA-ase may be stronger in tumor than in normal. Is that correct?

Dr. Naora: The combination of DNA and basic protein seems to be the important factor in stability. DNA-ase does not seem to be important in the maintenance of DNA.

Dr. Nakahara (Concluding): Time passes quickly when one is enjoying oneself, and like all other good things this conference will have to end.

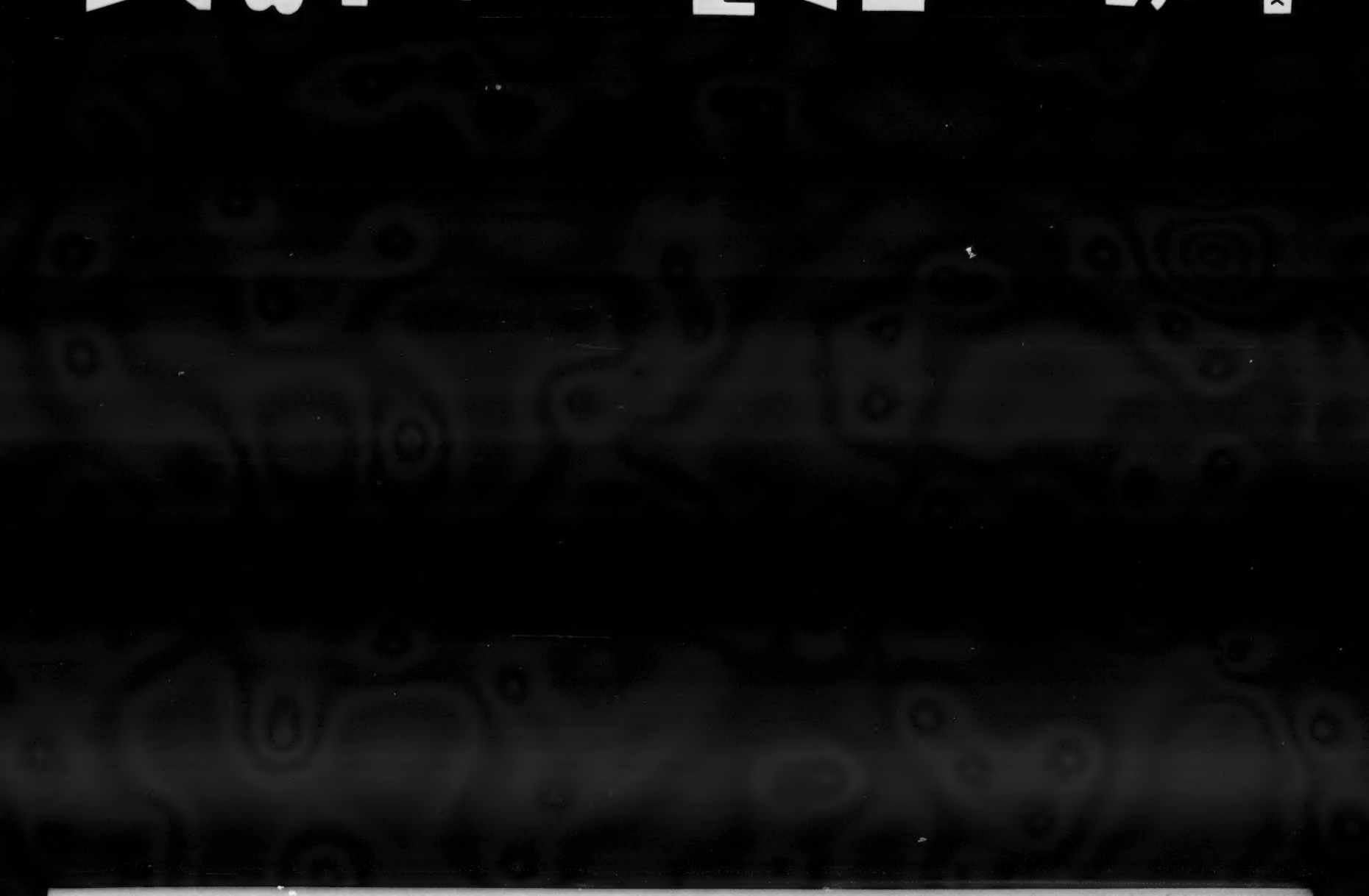
Thanks to the kind cooperation of Dr. Greenstein, this conference has been most stimulating and profitable. It will go down in the annals of cancer biochemistry in Japan as a big red letter event. Thank you, Dr. Greenstein, and thank you all.

The meeting is adjourned.

A New Honorary Member 新名誉会員

The following new Honorary Member of the Japanese Cancer Association was elected by the Executive Committee of the Association on October 26, 1956.

Dr. Jesse P. Greenstein, Chief of the Laboratory of Biochemistry, National Cancer Institute, Bethesda, Maryland, U. S. A.



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